Behavioural analysis of mouse mutants with altered neuronal and glial genes

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Table of contents

1. Introduction

1.1 The central and peripheral nervous system

The nervous system of mammals is made up of two parts which are the central nervous system (CNS) with the brain and the spinal cord and the peripheral nervous system (PNS), which covers all the parts of the nervous system except of the brain and the spinal cord. For all mammals, the brain is the control centre of nearly all physiological body functions and controls reactions, movements and behavioural responses. Additionally, the spinal cord is able to mediate stimulus reactions arriving from or leading to the brain. Both parts of the CNS actively control the integration of information and the coordination of activity of all body parts of animals. Conversely, the PNS is responsible for the connection of the CNS to the limbs and organs and for the monitoring of the whole internal and to some extent, external environment.

Anatomically, the brain is divided into five parts. These parts are the telencephalon (cerebral cortex, basal ganglia nuclei, amygdala, hippocampus, lateral ventricles), the diencephalon (thalamus, hypothalamus, pituitary gland, third ventricle), the mesencephalon (tectum, cerebral peduncle) and the rhombencephalon, separated into the metencephalon (pons, cerebellum) and the myelencephalon (medulla oblongata). Out of the medulla oblongata, the spinal cord leads through the vertebral column.

The brains of all vertebrates are composed primarily of two broad classes of cells: neurons and glial cells (neuroglia), while in the spinal cord of the vertebrates, the gray matter, in the centre of the cord, consists of cell bodies of interneurons and motor neurons. Additionally, it also consists of neuroglia cells and unmyelinated axons. The white matter is located outside of the gray matter and consists almost totally of myelinated motor, sensory axons and glial cells. In general, glial cells can be classified as astrocytes, microglia and oligodendrocytes.

1.1.1 Neurons

All neurons consist of a cell body, one axon and multiple dendrites, but differ in size and shape from uni-polar to multi-polar. They are electrically excitable cells, able to process and transmit information by electrical and chemical signals. The process of chemical signalling takes place at synapses, which connect a neuron to other cells. Neuronal connections in the brain are called networks or neuronal systems. Depending on their specialization, neurons can be separated into different groups: sensory neurons, motor neurons, local interneurons and principal projecting neurons. The first group reacts to sensory inputs as touch, sound,

light and numerous other stimuli, while the second group receives signals from the brain and spinal cord and processes these signals in order to cause muscle contractions. The third group, the interneurons, connects neurons to other neurons within the same region or between other regions of the brain and spinal cord, while the last group exists of neurons which axons project out of the region in which their cell somas lie to carry information to another region. With their axons, neurons are able to conduct electrical signals (nerve impulses) from the cell body to the axon terminals (synapses), where a small space (the synaptic cleft) is located. This synaptic cleft is the distance between a neuronal axon terminal (presynaptic) and a target cell (postsynaptic). The synapses are the locations, where chemical signals are initiated. The release of neurotransmitters in the synaptic cleft leads to a signal transmission from neuron to neuron. The combination of neurons of all neurotransmitter systems and their network of synapses manage all different brain functions, simple motor coordination as well as emotion, cognition or addiction processes.

1.1.2 Neuroglia

Neuroglia cells are non-neuronal cells in the brain which form myelin, and provide support and protection for neurons. Additionally, they have a central role in promoting the formation of synapses and modulation of synaptic activity. Astrocytes, microglia and oligodendrocytes are included in the group of neuroglia. Glial cells are the most abundant cells in the brain.

1.1.2.1 Astrocytes

Originally, astrocytes received their name because of their star-like cell form, but their shape can differ as well. They can normally be found between neurons and blood vessels and form complex networks with contacts to each other through gap junctions. By morphological criteria, two different types of astrocytes can be distinguished: fibrous and protoplasmic astrocytes (Lazzarini et al., 2004). Fibrous astrocytes can mainly be found in the white matter, while the protoplasmic type is the prevalent form in the grey matter. All astrocytes express glial fibrillary acidic protein (GFAP) (Bignami et al., 1972), cell adhesion molecules (CAMs), fibronectin, laminin and growth factors. One subtype of astrocytes originate are radial glia cells, which constitute a stem cell niche for glia cells in the developing CNS (Gotz and Steindler, 2003). These radial glia cells provide a scaffold for the migration of neural cells in the developing brain and resemble precursors during neurogenesis in adulthood (Noctor et al., 2001). Recently, it could be shown that astrocytes are able to respond to neurotransmitters released during synaptic activity, with an elevation of their intracellular Ca^{2+} concentration.

Additionally, astrocytes can release chemical transmitters, including glutamate, in response to elevations of the intracellular Ca^{2+} concentration (Santello and Volterra, 2009).

1.1.2.2 Microglia

Microglia cells are the most wide-spread cells all over the CNS and execute a diversity of functions. Most importantly, they work as macrophages inside the brain, defend the CNS against microorganisms and are capable of phagocytosis. Infections and inflammatory processes in the CNS cause an activation of microglia. Activated cells are able to secrete cytokines and other pro-inflammatory substances, which on one hand assist in repairing damage, but on the other hand, can lead to neuronal cell death (Dringen, 2005)

1.1.2.3 Oligodendrocytes and oligodendrocyte precursor cells (OPC)

Oligodendrocytes and Schwann cells are small cells with processes, which use those processes to insulate axons. Both types of cells have in common to build myelin sheaths wrapping the axons. Oligodendrocytes are responsible for the myelination in the CNS, while Schwann cells are in charge of this process in the PNS (Kandel, 1995). One important difference between the two is the quantity of ensheathing. One oligodendrocyte is able to encase an average of 15 axons in the CNS, while in the PNS one single Schwann cell ensheathes only one single axon (Kandel, 1995). The wrapped myelin sheath increases the conduction velocity and offers the possibility to transfer information over long distances.

Oligodendrocytes develop from precursor cells of the neuroepithelium and migrate from the ventricular (VZ) and the subventricular zone (SVZ) into grey and white matter. Here, these oligodendrocyte precursor cells (OPCs) contact the axonal tracts in early stages of development (Marshall et al., 2003) and become post-mitotic. Neurons and astrocytes release factors which lead to the differentiation of OPCs. Over the period of their maturation different transcription factors, proteins and lipids are expressed, which make it possible to characterize single maturation phases. This oligodendrogenesis starts with the stem cells, progress to migrating precursors, early precursors, late precursors, immature oligodendrocytes and finally reaches the stage of mature oligodendrocytes. Important in the early maturation phase of OPCs is the expression of the following antigens A2B5 (ganglioside antigen), NG2 (nerve-glia antigen 2) and PDGFα-receptor (platelet derived growth factor). When OPC differentiate into mature oligodendrocytes, these markers are downregulated and typical myelin proteins, such as MAG (myelin associated glycoprotein), MOG (myelin oligodendrocyte glycoprotein), and PLP (proteolipid protein) are upregulated.

It was found that OPCs express voltage-gated channels, glutamate and GABA receptors (Salter and Fern, 2005), but these receptors are lost after differentiation to oligodendrocytes.

Additionally, the presence of delayed rectifying K^+ channels and voltage-activated Na^+ channels was shown (Kettenmann et al., 1991).

1.2. The endocannabinoid system (ECS)

1.2.1 The endocannabinoid system and the cannabinoids

Since 3000 B.C., the hemp plant *Cannabis sativa* and its extracts have been known and used not only for the production of textiles but also for their healing and exhilarating effects. These effects stimulated scientists in the 19th century to start investigating the psychoactive ingredients of *Cannabis*. The Irish physician O'Shaughnessy played a central role in this newly developing research field by bringing *Cannabis* to Europe after his long visit to India, where he was able to observe its therapeutical use (Di Marzo, 2006). Since then, scientists identified more than 460 different compounds of the plant *Cannabis sativa.* Interestingly, only 60 of these compounds belong to the group of cannabinoids. In 1964 Gaoni and Mechoulam found that Δ^9-tetrahydrocannabinol (Δ^9-THC) (Fig.1) is the main compound, which accounts for the psychotropic effects of *Cannabis,* but the underlying mechanisms have been unknown for a long time (Gaoni and Mechoulam, 1964).

In addition to THC, two other non-psychotropic compounds were characterized, cannabidiol (CBD) and cannabinol (CBN) (Mechoulam et al., 1970). At the early stage of cannabinoid research, cannabinoids were seen just as psychoactive compounds isolated from *Cannabis sativa*. In 1990, the first "THC" cannabinoid receptor (CB1) (Matsuda et al. 1990) and shortly afterwards its endogenous ligands were identified in the mammalian brain (Devane et al., 1992). These endogenous ligands were named endocannabinoids and belong to a family of lipid messengers derived from fatty acids. By now, two main endogenous cannabinoids are known, anandamide (arachidonoyl ethanolamide, AEA), which was identified in 1992 (Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG), found in 1995 (Mechoulam et al., 1995; Sugiura et al., 1995). To date, a few more related endogenous lipids, acting as endocannabinoids, have been discovered, such as 2-arachidonoyl-glyceryl-ether (noladin ether) (Hanus et al., 2001), O-arachidonoyl-ethanolamine (virodhamine) (Porter et al., 2002) and N-arachidonoyl-dopamine (Huang et al., 2002). All of these fatty acids share the attribute that they derive from arachidonic acid. Different from other neurotransmitters, these polyunsaturated fatty acids cannot be stored in vesicles and are mostly synthesized "on-demand" from membrane phospholipid precursors (Massa and Monory 2006), which means that their synthesis depends on distinct stimuli. Until today anandamide and 2-AG (Di Marzo et al., 2004) are the best-studied endocannabinoids. Parallel to the discovery of the main endocanna-

binoids, Munro et al. (1993) were able to clone the second cannabinoid receptor (CB2). The endocannabinoid anandamide is promiscuous and e.g., is able to bind and activate other kind of receptors, such as the vanilloid receptor type 1 (TRPV1) (Zygmunt et al., 1999). Additionally, anandamide is able to inhibit the TASK-1 K^+ channel (Maingret et al., 2001), a potassium channel responsible to set the resting membrane potential. The existence of non-CB1/CB2 cannabinoid receptor has been frequently discussed, but their genes have not yet been cloned (Sagan et al. 1999). Recent findings suggest that the G-protein coupled receptor GPR55 is a putative third cannabinoid receptor, with a signalling distinct from CB1 and CB2 (Lauckner et al., 2008). De Petrocellis and Di Marzo reviewed in 2010 the effects of the endocannabinoids, phytocannabinoids and synthetic cannabimimetics on this orphan G-protein coupled receptor and could point, how different these effects were depending on the drug used and the tissue expressing this receptor (De Petrocellis and Di Marzo, 2010).

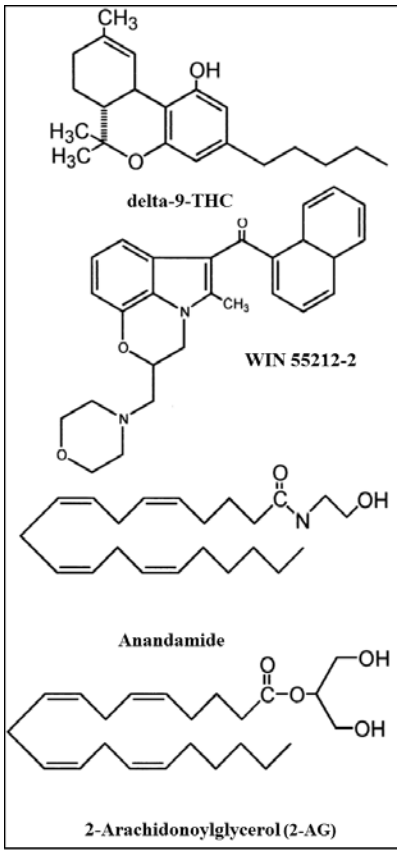

Fig. 1: Chemical structures of cannabinoid receptor agonists. The phytocannabinoid THC, the synthetic cannabinoid WIN 55212-2 and the endocannabinoids anandamide and 2-AG are shown (modified from Iversen, 2003).

This discovery of the CB1 receptor, and the recognition of a series of endogenous cannabinoids (Fig. 1) acting as ligands for these receptors, stimulated the research on *Cannabis* in

the past two decades. Thus, it was possible to reveal a hitherto mostly unknown physiological control mechanism – the endocannabinoid system (ECS), which is constituted by the different cannabinoid receptors, the endocannabinoids and their enzymes for biosynthesis and degradation (Piomelli, 2003; Kano et al., 2009). The two main endocannabinoids differ in their pathways of synthesis, but the synthesis is generally activated in response to a depolarization-induced increase in the concentration of intracellular calcium (Di et al., 2005), but it can also be stimulated independent from calcium influx by metabotropic glutamate receptors (Varma et al., 2001). Accordingly, metabotropic glutamate receptors and depolarization represent two ways of triggering the endocannabinoid synthesis. 2-AG is generated from diacylglycerol (DAG) by the enzyme *sn*-1-selective diacylglycerol lipase (Fig. 2). In 2003, two DAG lipase isozymes, DAGL-α and DAGL-β, were cloned (Bisogno et al., 2003). The intracellular second messenger DAG, which activates protein kinase C, is generated from phosphoinositides by a phosphoinositide-specific phospholipase C or from phosphatidic acid by phosphatidic acid phosphohydrolase (Bisogno et al., 2003). The second important endocannabinoid, anandamide, is synthesized through a Ca^{2+}-dependent *trans*-acylase and the enzymatic hydrolysis of the membrane precursor N-arachidonoyl phosphatidylethanolamide (NAPE). Schmid et al. could already show in 1983 that this hydrolysis is conducted with the enzyme phospholipase D. The generation of NAPE is accomplished by the enzymatic transfer of arachidonic acid in the *sn*-1 position in phosphatidylcholine to the amide group of phosphatidylethanolamide (Di Marzo et al., 1994; Cadas et al., 1997). Recently the possibility emerged for other parallel pathways for the synthesis of anandamide do exist (Sun et al., 2004; Liu et al., 2000).

To date, the knowledge about the molecular identity of the uptake process of endocannabinoids is limited. It is proposed that this process is mediated by a specific transporter or facilitatory carrier protein across the cell membrane. Until now it has not yet been possible to exactly identify the transporter for both endocannabinoids. Fu et al. were recently able to describe a partly cytosolic variant of the intracellular anandamide degrading enzyme fatty acid amide hydrolase (FAAH), named FAAH-like anandamide transporter (FLAT), which lacked amidase activity but bound anandamide with low micromolar affinity and facilitated its translocation into the neurons (Fu et al., 2011). The degradation of endocannabinoids after reuptake is induced by intracellular enzymatic degradation. Similar to the different synthesis pathways of the main endocannabinoids, the degradation follows two different pathways as well (Fig. 2). The hydrolysis of anandamide is catalysed by FAAH (Giang and Cravatt, 1997), while the hydrolysis of 2-AG is catalysed by monoacylglycerol lipase (MAGL) (Dinh et al., 2002a; Dinh et al., 2002b; Saario et al., 2004).

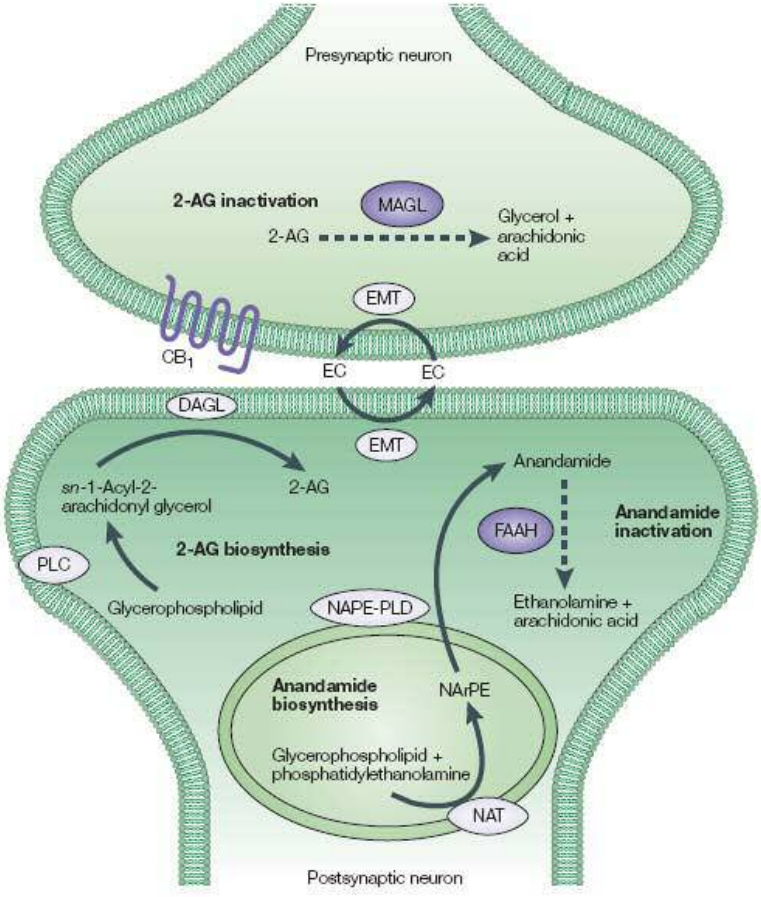

Fig. 2: Pathways involved in the synthesis and degradation of endocannabinoids and their most likely subcellular localization. The enzymes for 2-arachidonoylglycerol (2-AG) biosynthesis, the phospholipases C (PLC) and the *sn*-1-selective diacylglycerol lipases (DAGLs) seem to be mostly localized on the plasma membrane. The DAGLs are mainly located on postsynaptic neurons, whereas the monoacylglycerol lipase (MAGL) for 2-AG inactivation is localized in presynaptic neurons, which supports its possible role as retrograde messenger at presynaptic CB1 receptors. The anandamide biosynthetic enzymes *N*-acyltransferase (NAT) and *N*-acylphosphatidyl-ethanolamine-specific phospholipase D (NAPE-PLD) and the inactivating enzyme fatty acid amide hydrolase (FAAH) are all located on intracellular membranes. FAAH seems to be mainly present on neurons postsynaptic to CB1 receptors, indicating that anandamide acts principally on these neurons. However, whether NAT and NAPE-PLD are pre- or postsynaptic is not known. Finally, a putative endocannabinoid membrane transporter (EMT) seems to facilitate both endocannabinoid release and re-uptake, and might be localized on both pre- and postsynaptic neurons. NArPE, *N*-arachidonoylphosphatidyl-ethanolamine (modified from Di Marzo et al., 2004).

The ECS is an important neuromodulatory system in the brain (Piomelli, 2003; Marsicano and Lutz, 2006). Several neuronal functions are under the control of the CB1 receptor and its endogenous ligands. The expression of the CB1 receptor is wide-spread over different brain regions and occurs in distinct neuronal subpopulations. The distinct distribution of the CB1 receptor reflects the complexity and the variety of functions of the ECS in brain functions. Endocannabinoids can act as retrograde transmitters at the synaptic level in many brain regions.

This means that postsynaptically released endocannabinoids subsequently act as retrograde messengers. They bind to presynaptic CB1 receptors. This activation of the CB1 receptor results in an inhibition of voltage-sensitive calcium channels and an activation of potassium channels (Wilson et al., 2001). This causes a membrane depolarization, inhibits the release of neurotransmitters (Marsicano and Lutz, 2006) and affects neuronal cell excitability (Marsicano et al., 2003). Additionally, the stimulation of the CB1 receptor can also exert regulation of various forms of long-term synaptic plasticity (Marsicano and Lutz 2006). Depending on the regions and neuronal circuits involved, the mechanisms of the endocannabinoid-controlled synaptic modulation can be different. On the one hand, the CB1 receptor can predominantly be found at pre-synaptic terminals of neurons (Fig. 2). Their activation can lead to the decrease of the release of several neurotransmitters such as γ-aminobutyric acid (GABA), glutamate, serotonin or dopamine (Szabo and Schlicker, 2005; Freund et al., 2003). On the other hand, endocannabinoids are also able to act in an autocrine or a paracrine manner (Marsicano and Lutz, 2006).

The ECS can modulate several physiological functions, such as pain perception (Iversen and Chapman, 2002), motor activity (Van der Stelt and Di Marzo, 2003) and memory processing (Wotjak, 2005; Marsicano et al., 2002; Lichtman et al., 2002). Furthermore the ECS influences cardiovascular and respiratory functions (Mendizabal and Adler-Graschinsky, 2003) and possesses antiproliferative actions in tumour cells (Bifulco and Di Marzo, 2002). Additionally, the ECS takes part in the modulation of immune and inflammatory responses (Massa et al., 2004; Klein et al., 2003) and in neuroprotection (Panikashvili et al, 2005; Panikashvili et al, 2001). For example, this neuroprotection is based on the CB1 receptor mediated prevention of excitotoxicity (Marsicano et al., 2003). During an excitotoxic insult, neuronal damage is generated by a very high glutamate release in the hippocampus. In an *in vivo* model of excitotoxity, the application of kainic acid causes excessive neuronal activity by stimulating kainate receptors, which are endogenously activated by glutamate. Thus, kainic acid induces both excitotoxic and epileptogenic events. Loss of the CB1 receptor increased the susceptibility to this experimental excitotoxity. Application of kainic acid also induces increased levels of anandamide in wild-type mice (Marsicano et al., 2003). Consequently, the inhibition of endocannabinoid reuptake protects treated animals from seizures via the activation of the CB1 receptor, which finally leads to a reduced glutamate release (Marsicano et al., 2003, Lutz 2004, Monory et al., 2006).

Furthermore, endocannabinoids are involved in the regulation of hypothalamic hormone release, which lead to the modulation of food intake and the hypothalamic-pituitary-adrenal axis (Steiner et al., 2008; Wenger and Moldrich, 2002). Cannabinoids and endocannabinoids are also known to induce potent effects on movement mediated by the CB1 receptor (Sañudo-Peña et al., 2000). The brain shows a distinct pattern of expression of the CB1 re-

ceptor being highly concentrated in areas such as the basal ganglia and cerebellum, which control motor behaviour. The major effect of cannabinoids in movement is hypoactivity and catalepsy (Ferrari et al., 1999). However, cannabinoid receptor agonists can also induce biphasic effects on movement, depending on time and dose of application. An increase in motor activity has been associated with relatively low doses or can be seen immediately after administration of higher doses. Observed at a later time point after administration, high doses of cannabinoid receptor agonists lead to an inhibition of movement and produce catalepsy (Hollister, 1986). Additionally, a study of CB1 receptor knock-out mice reported a decrease in the activity of these animals, suggesting an activating role of the CB1 receptor on movement (Zimmer et al., 1999). Bilkei-Gorzo et al. reported in 2005 that the ECS participates in the age-related decline of learning and memory functions. Recent publications of this group could show that especially the CB1 receptor activity on hippocampal GABAergic neurons protects from pyramidal cell degeneration and neuroinflammation (Albayram et al.; 2011). The cannabinoid system is subject to characteristic age-related changes. It was shown that old animals, when compared to young animals, had reduced CB1 receptor densities and mRNA expression levels in many brain areas, most prominently in the basal ganglia and in the cerebellum (Romero et al. 1998).

The CB1 receptor is predominantly found at the presynaptic terminals of two neuronal populations, i.e. on inhibitory GABAergic and excitatory glutamatergic neurons. By using different transgenic mouse lines, Häring et al. (2011) investigated the impact of CB1 receptor inactivation in glutamatergic or GABAergic neurons on investigatory behaviour and could show that exploratory behaviour is accurately balanced in a social and a non-social context by the ECS via CB1 receptor activation on cortical glutamatergic and GABAergic neurons. The deletion of the CB1 receptor from cortical and striatal GABAergic neurons increased the exploratory behaviour of the mice, while the CB1 receptor deletion from cortical glutamatergic neurons resulted in a decreased exploration. Exogenous cannabinoids, through activation of CB1 receptor, seem also to be able to regulate emotional behaviour. Δ^9-THC and other CB1 receptor agonists may induce biphasic effect in models of anxiety. Low doses of cannabinoids usually induce an anxiolytic-like effect, whereas higher doses cause an anxiogenic response (Berrendero and Maldonado, 2002; Viveros et al., 2005). Furthermore, CB1 receptor knock-out mice have an increase in the basal level of anxiety in the light-dark test (Martin et al. 2002). Recently, it was shown that the genetic deletion of CB1 receptor in D1-expressing neurons was able to affect the emotional behaviour in mice in a highly selective manner, which indicates that there might be a cross-talk between dopaminergic D1 receptors and the ECS in terms of controlling a negative effect in the emotional behaviour in mice (Terzian et al., 2011). This indicates that the modulation effect of the ECS on the dopaminergic system might not only have an effect on addiction properties but also on emotional properties.

As Maldonado et al. showed in 2006, the ECS is involved in the common neurobiological mechanism underlying drug addiction. The ECS is also crucial in the addictive effects of nicotine and mediates the nicotine relapse induced by associated environmental stimuli (Maldonado et al., 2006). These effects of the ECS on the rewarding properties of nicotine are related to the modulation of the extent to which nicotine activates the mesolimbic dopaminergic pathway and leads directly or indirectly to the increase of dopamine in brain areas, such as the nucleus accumbens and ventral tegmental area. Additionally, a conditional deletion of CB1 receptor in forebrain principal neurons or GABAergic neurons prevented cocaine-induced extracellular signal-regulated kinase activation in the dorsal striatum and nucleus accumbens. Evidence could be provided for the role of the ECS in regulating neuronal circuits important for long-lasting effects of cocaine, which could act on CB1 receptor located on terminals of striatal medium spiny neurons (Corbille et al., 2007).

1.2.2. Distribution of cannabinoid receptors in the brain

Both known cannabinoid receptors belong to the class of seven transmembrane receptors coupled to $G_{i/o}$ proteins (Fig. 3). Their activation is linked to the inhibition of adenylyl cyclase activity (Howlett et al., 1988). The CB1 receptor shows a great abundance and is widely distributed in many regions of the CNS. The CB2 receptor is mainly found in immune and blood cells, where it may participate in regulating immune responses (Howlett et al., 2002; Pereira et al., 2009). Some publications have shown that CB2 receptor is present in the CNS (Gong et al., 2006; Van Sickle et al., 2005), in the endocrine pancreas and in bones (Juan-Pico et al. 2006, Ofek et al. 2006) as well. CB1 and CB2 receptors share relatively small sequence homology, only 44% at the protein level and 68% in the transmembrane domains, which are thought to contain the binding sites for cannabinoids (Lutz, 2002). Δ^9-THC, anandamide and 2-AG (Fig. 1) activate both receptors, but synthetic selective ligands for each receptor were developed as well.

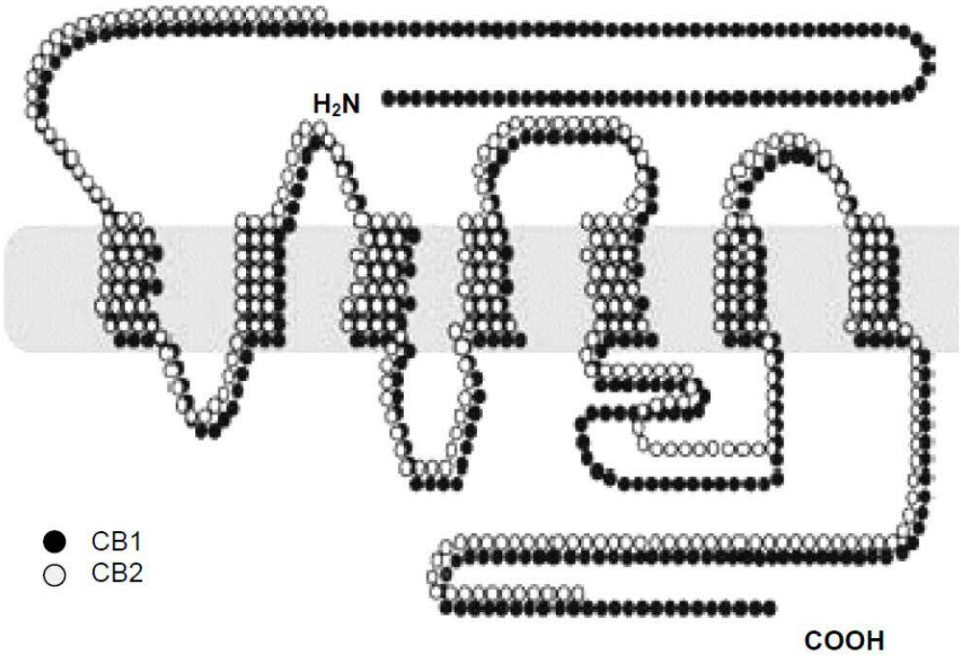

Fig. 3: Schematic presentation of cannabinoid receptor type 1 (CB1) and type 2 (CB2). Both belong to the class of seven transmembrane receptors coupled to $G_{i/o}$ proteins.

In the CNS, CB1 receptor is by far the most abundant G-protein coupled receptor. CB1 receptor is expressed strongly in the basal ganglia, cerebellum, and hippocampus accounting for the well-known effects of *Cannabis* on motor coordination and short-term-memory processing (Marsicano and Lutz 1999). Likewise, CB1 receptor is expressed at high concentrations in the dorsal primary afferent spinal-cord regions, which are important in pain pathways, whereas it is expressed at low concentrations in the brainstem controlling many autonomic functions. This may account for the lack of *Cannabis* induced acute fatalities. Therefore, the high number of effects of *Cannabis* is due to the presence of CB1 receptor in regions that control diverse functions. These findings are supported by studies on CB1 receptor knock-out mice, in which the effects normally induced by cannabinoids are abolished (Zimmer et al., 1999; Ledent et al., 1999; Marsicano et al., 2002).

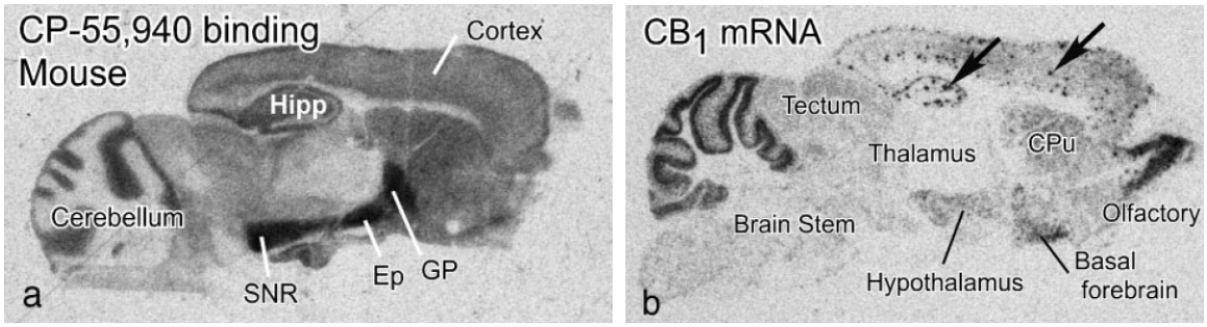

Fig. 4: CB1 expression in different brain regions. Autoradiographs show cannabinoid receptor binding (a) and CB1 mRNA expression (b) in sagittal sections from the mouse. Receptor binding of [3H] CP55940, a high-affinity agonist, shows high levels of receptors in the basal ganglia, cerebellum, hippocampus (hipp), and cerebral cortex (a). Cells expressing CB1 mRNA are shown in a similar plane of section (b). The high levels of binding in many areas [cerebellar molecular layer, globus pallidus (GP), entopeduncular nucleus (Ep), substantia nigra pars reticulata (SNR), and dentate gyrus molecular layer] are on axons of cells expressing mRNA in afferent areas, such as the caudate putamen (CPu). Some cells in cortex and hippocampus express extremely high levels of CB1 message (arrows in b) (modified from Howlett et al., 2002).

The cloning of the CB1 receptor was achieved in several different vertebrates, including the human, cat, rat, mouse, and in different birds, amphibians and fish (McPartland et al., 2006; Lutz, 2002). Among these different species, considerable similarities were found. Thus, the human CB1 protein shares 97% identity with the CB1 receptor of mouse, 84% with the amphibian CB1 receptor, 72% with the fish $CB1_A$ and 59% with the fish $CB1_B$. This high level of identity suggests that the results obtained with mice are probably (at least to a degree) applicable to humans.

As mentioned above, CB1 receptors are the most abundant G-protein-coupled receptors in the mammalian brain, their densities being similar to levels of γ-aminobutyric acid (GABA) - and glutamate-gated ion channels (Howlett et al., 2004). It is important to know that the CB1 receptor is expressed at different levels in different neuronal subpopulations. High levels of CB1 protein and CB1 mRNA are specifically present in cortical GABAergic interneurons, where they are able to mediate endocannabinoid-dependent inhibition of GABA release (Marsicano and Lutz, 1999, 2006; Freund et al., 2003). Additionally, CB1 receptor is expressed in other neuronal subpopulations, including, amongst others, glutamatergic cortical principal neurons (Marsicano and Lutz, 1999; Monory et al., 2006).

Furthermore this research on CB1 receptor revealed details of CB1 distribution and its correlation with the behavioural effects of cannabinoids (Marsicano and Lutz, 1999; Hermann et al., 2002, Häring et al, 2007). The density of CB1 receptor is very high in the basal ganglia (substantia nigra, globus pallidus, entopeduncular nucleus and lateral caudate putamen), and in the molecular layer of the cerebellum (Fig. 4). This high CB1 receptor density in the

basal ganglia is consistent with the effects of cannabinoids on spontaneous locomotor activity and catalepsy in rodents (Compton et al., 1992). In addition, very high CB1 receptor expression levels are present in distinct areas of the hippocampus, particularly in the CA pyramidal cell layers and the dentate gyrus, and in layers I and IV of the cortex. The high densities in these brain regions point at an involvement of cannabinoids in the regulation of cognition and memory.

1.3. The dopaminergic system

1.3.1. A general overview

Dopamine (Fig. 5) has been known for approximately 100 years, first synthesized in 1910 by Barger and Ewens, but was not characterized as a neurotransmitter until 1958 by Arvid Carlsson. It is a catecholamine-class neurotransmitter and influences CNS function in many ways. The biosynthesis of dopamine starts with hydroxylation of the amino acid L-tyrosine to L-DOPA (3,4 dihydroxyl-L-phenylalanine) via the enzyme tyrosine 3-monooxygenase also known by its former name tyrosine hydroxylase. Afterwards L-DOPA is decarboxylated by aromatic L-amino acid decarboxylase or DOPA decarboxylase. After its biosynthesis, dopamine is packed into vesicles inside neurons. In response to a presynaptic action potential, these vesicles fuse with the cell membrane and dopamine is released (Fig.6). In order to terminate neurotransmission, dopamine can be taken up into the neuron with the help of a specific dopamine transporter (DAT) and it will be degraded by enzymes intracellularly or will be recycled.

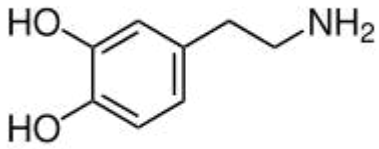

Fig. 5: Chemical structure of dopamine

In the brain, dopamine activates two groups of receptors, which belong to the class of metabotropic G-protein coupled receptors, and can be found all over the central nervous system. D1 and D5 receptors represent the D1-like family and their receptor activation is coupled to the G protein $G_{\alpha s}$. $G_{\alpha s}$ subsequently activates adenylyl cyclase by increasing the intracellular concentration of the second messenger cyclic adenosine monophosphate

(cAMP). An increased cAMP level in neurons can be excitatory and can then induce an action potential by modulating the activity of ion channels. D2, D3 and D4 receptors belong to the so called D2-like family. Their activation is directly coupled to the G-protein $G_{\alpha i}$, which increases phosphodiesterase activity. Phosphodiesterases degrade cAMP and can therefore produce an inhibitory effect in neurons. Additionally, this D2-like family is known to inhibit adenylyl cyclase via Gi/o, respectively (Nishi et al., 2011)

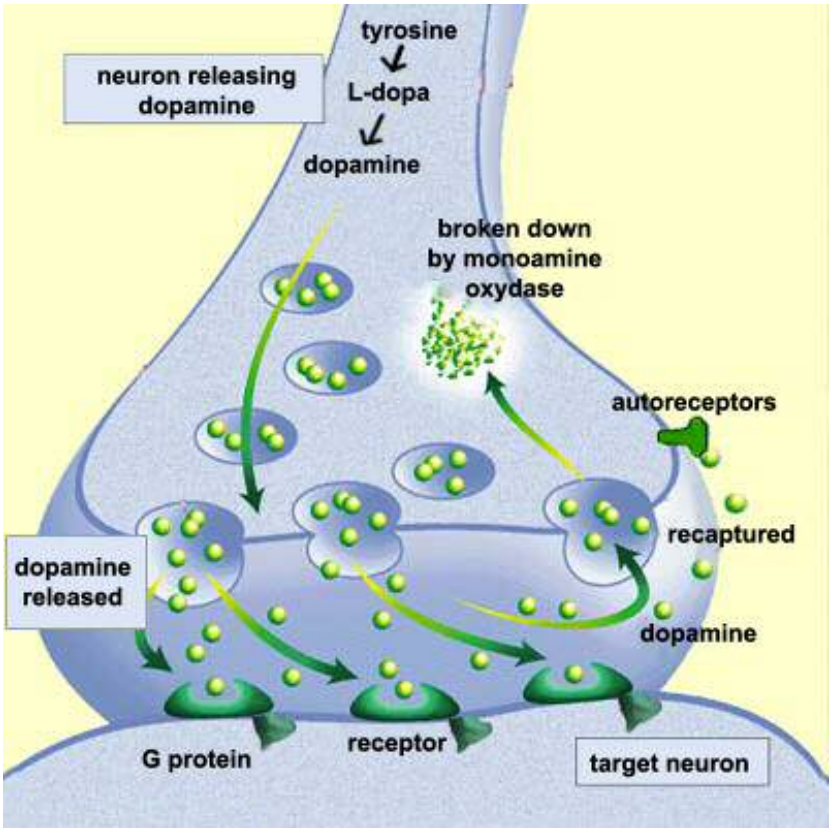

Fig. 6: Dopamine biosynthesis and degradation. Dopamine is biosynthesized first by the hydroxylation of the amino acid tyrosine to L-DOPA via the enzyme tyrosine 3-monooxygenase (tyrosine hydroxylase) and then by the decarboxylation of L-DOPA by aromatic L-amino acid decarboxylase. In neurons dopamine is packaged after synthesis into vesicles, which are then released into the synapse in response to a presynaptic action potential. In most areas of the brain, including the striatum and basal ganglia, dopamine is inactivated by reuptake via the dopamine transporter (autoreceptor) and then enzymatic broken down by monoamine oxidase (modified from www.thebrain.mcgill.ca).

It is known that dopamine has several important roles in behaviour and cognition, motor activity, motivation and reward, addiction processes, attention and learning. These different roles are attributable to its distribution and production in the brain. Accordingly, dopaminergic neurons are present in the ventral tegmental area (VTA) of the midbrain projecting to the ventral striatum (nucleus accumbens) and the pre-frontal cortex. In substantia nigra pars compacta, the neurons project to the dorsal striatum (caudate-putamen) (Fig.7).

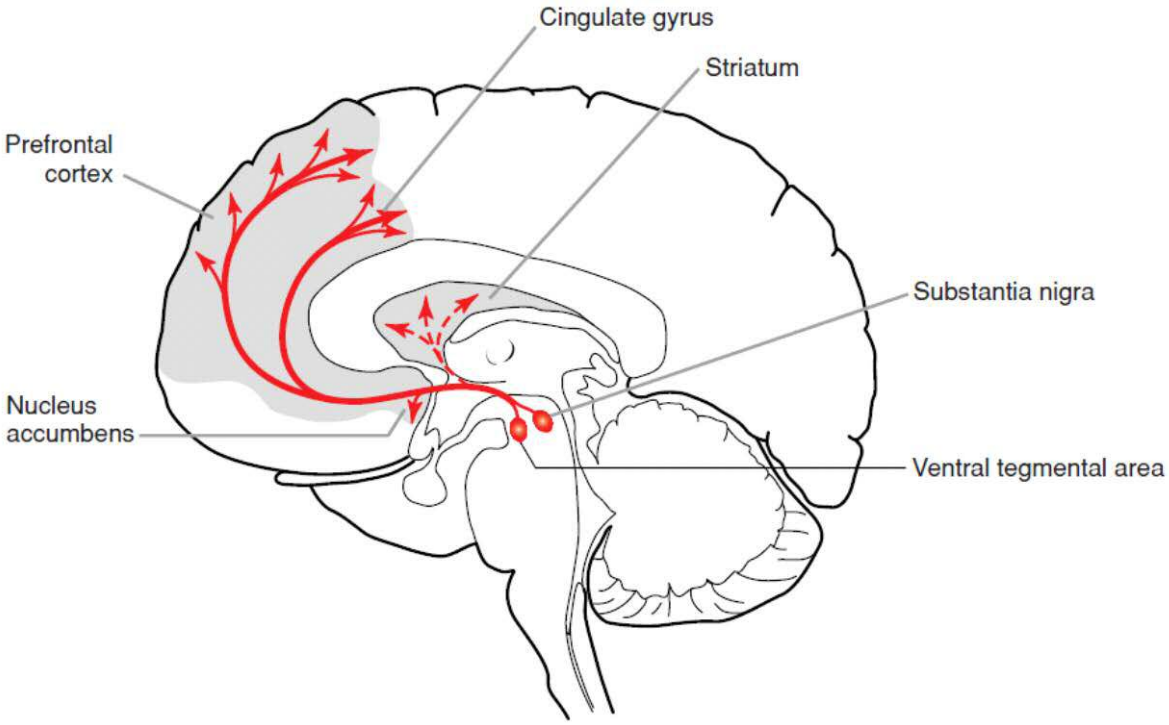

Fig. 7: Dopaminergic pathways to the forebrain. Illustrated are projections from the ventral tegmental area to the nucleus accumbens and to the prefrontal cortex and projections from the substantia nigra to the dorsal striatum (modified from Hyman et al., 2006)

The dopaminergic pathway to the striatum or nigro-striatal pathway is important for motor behaviour and for the learning of complex motor behaviour (Döbrössy and Dunnett, 2001). The dopaminergic pathway to the ventral striatum or so-called mesolimbic dopamine pathway is involved in motivation and learning (Dickinson, 1994). Both D1 and D2 receptors are highly expressed in the striatum. The dopaminergic pathway to the pre-frontal cortex, where D1 receptors are also expressed, modulates cognitive behaviour (Goldman-Rakic, 1992). Depending on the drug of abuse, the mechanism of the addiction processes in the dopaminergic system (DAS) differs. Several groups of compounds that produce different pharmacological effects can lead to addictive behaviour, including opioids, psychostimulants such as cocaine or amphetamine, cannabinoids, alcohol and nicotine. The initial mechanism of action of these distinct drugs implicates different neurochemical targets (Hyman and Malenka, 2001). Despite these differences each compound produces a neural dysregulation, which involves similar neurochemical and neuroanatomical pathways (Nestler, 2004). For example, cocaine blocks the DAT, which is located on the presynaptic membrane of dopaminergic neurons, and thereby increases a blockage of synaptic dopamine. In contrast, amphetamines enter dopamine neurons via DAT and then interact with the vesicular monoamine transporter (VMAT) inside the cell and therefore induce the release of dopamine from the presynaptic

terminal (Hyman et al., 2006). Alternatively, nicotine acts directly on nicotinic acetylcholine receptors (nAChRs) on dopaminergic neurons in the VTA and causes dopamine release in the nucleus accumbens. THC (cannabinoids) and endocannabinoids act via the CB1 cannabinoid receptor expressed in dopaminergic terminals, producing a release of dopamine (Hyman et al., 2006). Alternatively, cannabinoids and endocannabinoids can act through the modulation of GABA and glutamate inputs onto dopaminergic neurons in the VTA.

More than any other work on neurotransmitters, the research on dopamine and the dopaminergic system has extremely influenced the understanding of mental illnesses and was conductive to the development of modern psychopharmacology (Iversen and Iversen, 2007). An inequality in the balance of dopamine activity can cause brain dysfunction and diseases. For more than 30 years, scientists have studied the connections between dopamine dysfunction and several CNS diseases, such as schizophrenia, Parkinson's disease and drug addiction. Additionally, it is known that disruption of processes in the mesolimbic dopamine system heightens the distractibility in attention deficit hyperactivity disorder (ADHD).

1.4. Neuromodulatory functions of the endocannabinoid system

The ECS is able to interact with different other systems such as the GABAergic (Szabo et al., 1998; Chan et al., 1998), glutamatergic (Monory et al., 2006), serotonergic (Egashira et al., 2006) and dopaminergic system (DAS) (Herkenham et al., 1991; Maneuf et al., 1996) in specific brain areas. The basal ganglia and cerebellum exhibit a high density of the CB1 receptor (Herkenham et al., 1990, 1991; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998; Hohmann and Herkenham, 2000). Within the basal ganglia, the highest density of the CB1 receptor is found on axon terminals from neurons of the striatum and subthalamic nucleus that project to substantia nigra, globus pallidus and entopeduncular nucleus (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992). The high density of CB1 receptors in basal ganglia points at an involvement in motor activity. As expected, the presence of endocannabinoids has also been shown in these brain regions (Di Marzo et al., 2000). Other studies showed the presence of CB1 receptor mRNA transcripts in the caudate putamen (Mailleux and Vanderhaeghen, 1992; Marsicano and Lutz, 1999). This implied that synthesis of CB1 receptor happen in striatal GABAergic efferent neurons as well (Romero et al., 2002).

Activation of CB1 receptors on axon terminals in striatum and substantia nigra can inhibit GABA release (Maneuf et al., 1996) and at the same time increase the firing of dopaminergic neurons, because of the tonical inhibition of VTA dopamine neurons by GABAergic interneurons (Hyman et al., 2006). It was assumed that dopaminergic transmission can be affected indirectly by cannabinoids and their effects on GABAergic neurons (Romero et al., 2002). A

colocalization of the CB1 receptor was found with D1 or D2 dopamine receptors on projection neurons from the striatum (Herkenham et al., 1991; Hermann et al., 2002) and CB1 mRNA expression seems to be negatively controlled by nigral dopaminergic signalling (Lastres-Becker et al., 2001). It is accepted that CB1 receptors play a modulatory function on dopamine transmission, because of the indirect effects of cannabinoids, exerted through the modulation of GABA and glutamate inputs received by dopaminergic neurons. But there is still no proof that CB1 receptors are located in dopaminergic terminals in the major brain regions which receive dopaminergic innervation, such as caudate-putamen and nucleus accumbens or prefrontal cortex (Fernandez-Ruiz et al., 2010). But Lau and Schloss (2008) were able to show that the expression of cannabinoid CB1 receptors is not restricted to presynaptic terminals of serotonergic and dopaminergic neurons, but is also found on the neuronal cell bodies of cultured free-floating embryonic mouse stem cells.
In addition, other studies show that cannabinoids inhibit GABA transmission in inhibitory neurons in the substantia nigra pars reticulata (SNR), possibly by a presynaptic mechanism via the decrease of Ca^{2+} or the activation of potassium channels (Chan et al., 1998; Szabo et al., 1998, 2005). Cannabinoids are able to influence the excitatory glutamatergic transmission as well. In the striatum, electrophysiological studies showed that a Ca^{2+}-dependent K^{+}-evoked glutamate release is inhibited by cannabinoids (Köfalvi et al., 2005).

1.4.1. Interaction of the dopaminergic and endocannabinoid system

The research on the dopaminergic system (DAS) and on the endocannabinoid system (ECS) shows many analogies. This work tries to scrutinize those analogies. The density of the receptors of both systems in specific brain regions such as the striatum offers a start point and suggests that both systems are cross-connected in different functions.
Giuffrida et al. already showed in 1999 that interactions between ECS and DAS contribute to striatal signalling (Giuffrida et al.; 1999). As it is known nowadays, the basal ganglia (or basal nuclei) are a group of nuclei, situated at the base of the forebrain and are strongly connected with the cerebral cortex, thalamus and other brain areas. These basal ganglia are made up of the striatum which is composed of caudate and putamen, the globus pallidus existing of globus pallidus externa (GPe) and globus pallidus interna (GPi), the substantia nigra composed of both substantia nigra pars compacta (SNc) and substantia nigra pars reticulata (SNr), and the subthalamic nucleus (STN) (Fig. 105). The striatum is one of the key components of this forebrain system that also controls planning and the execution of motor behaviours. Excitatory signals, which were generated in the sensorimotor and limbic areas of the neocortex and in the thalamus, arrive to the striatum, are combined and spread to other

structures of the basal ganglia and to the substantia nigra. Until today, however, it is not completely understood, how the striatum arranges these processes that are mediated by the neurotransmitter glutamate. Cannabinoid receptors of the ECS are densely expressed in striatum (Herkenham, 1990). Compared to D1 receptors, their density is twice as numerous in this brain region. The activation of cannabinoid receptors has profound consequences on the electrophysiological properties of striatal neurons (Szabo et al., 1998), as well as on motor behaviours mediated by striatal projection systems (Sañudo-Peña et al., 1996).

Dopamine interacts with both groups of G-protein-coupled receptors, D1-like and D2-like, and therefore regulates essential aspects of striatal physiology (Graybiel et al., 1994). By administration of a D2-like agonist in striatum an eightfold stimulation of the endocannabinoid AEA outflow can be seen (Giuffrida et al., 1999). This suggests that the ECS participates in dopaminergic regulation of striatal function. D2-like receptors occupied by dopamine cause the release of AEA in striatum and possibly in other regions of the CNS, which participate in the control of movement and locomotion. By engaging the CB1 receptor, anandamide interferes with dopamine stimulation of motor activity, which is thought to be differently mediated by postsynaptic D2-like and D1-like receptors, based on the different expression of D1 receptors on direct output neurons and D2 receptors on indirect output neurons (Drago et al., 1994).

Not only locomotion is affected by DAS and ECS. Both systems are known to be involved in the control of addiction. Dopamine is commonly associated with the “pleasure system” of the brain. Δ^9-THC or nicotine leads directly or indirectly to the increase of dopamine in the shell of nucleus accumbens. The neurotransmitter dopamine increases locomotion and cannabinoids reduce locomotion. An overflow of dopamine in the brain leads to hyperactivity. At the same time it causes the release of anandamide, which decreases the hyperactive effect of dopamine. The same effect can be seen in brain regions responsible for anxiety e.g. the ventral striatum, where the density of CB1 and D1 receptors is high. Dopamine causes anxiogenic behaviour in animals while cannabinoids in low doses reduce anxiety in mice (Valjent et al., 2002). In a model of addiction for nicotine, which increases the release of dopamine, strongly facilitated effects were induced by administration of Δ^9-THC demonstrating the existence of a functional interaction between Δ^9-THC and nicotine (Valjent et al., 2002). The facilitation of THC-induced acute pharmacological and biochemical responses, tolerance and physical dependence by nicotine play an important role in the development of addictive processes caused in striatum. The density of the receptors of both ECS and DAS in this area implicates that both systems are involved in addiction.

Recent publications have shown that a systemic, an intranasal or an intra-accumbens local administration of the selective CB2 receptor agonist JWH133, can dose-dependently inhibit intravenous cocaine self-administration, cocaine-enhanced locomotion, and cocaine-

enhanced accumbens extracellular dopamine in wild-type and CB1 receptor knock-out mice, but not in CB2 receptor knock-out mice (Xi et al., 2011). This implies that brain CB2 receptors have the possibility to modulate cocaine's rewarding and locomotor-stimulating effects, likely by a dopamine-dependent mechanism. Already in 2005 Soria et al. evaluated the role of CB1 cannabinoid receptors in several aspects of cocaine reward, including acquisition, maintenance, and motivation to seek the drug and they showed that the CB1 receptor seems to have an important role in the consolidation of cocaine reinforcement, although they were not required for its acute effects on mesolimbic dopaminergic transmission.
These findings lead to the assumption that the interactions of ECS and DAS do not depend solely on their direct activation with their own agonists and antagonists, but additionally on an indirect activation by other drugs as well.

1.5. Nerve-Glia antigen 2 (NG2) and the NG2-EYFP mouse line

1.5.1. NG2 protein

The NG2 proteoglycan is a type 1-transmembrane glycoprotein and is expressed by many different immature and adult cell types in the nervous system including oligodendrocyte precursor cells, immature glia, subpopulations of perisynaptic glia, immature Schwann cells, pericytes, immature muscle cells and skeletal myoblast throughout development and in the adult brain (Karram et al., 2005). Cells, which express NG2 (NG2+ cells), can make up 5–10% of all glia in the developing and adult CNS. These cells can be found in white and grey matter. It seems that these cells have a continual turnover, as it was found that some of them are able to proliferate even in the adult CNS. But NG2 protein expression is down-regulated upon maturation of the cells (Trotter et al., 2010). A distinct subset of NG2+ glial cells in the hippocampus was reported to receive direct synaptic input from glutamatergic and GABAergic neurons (Bergles et al., 2000). Additionally, in 2005 Jabs et al. could show that glutamate receptor expressing cells, which are positive for NG2 as well, develop an unusual synaptic association with neurons. This implicates the possibility that NG2 glia act not only as a plastic progenitor pool for more differentiated cells, but may form a unique glial network as well. It is known that oligodendrocytes and astrocytes express distinct sets of connexin (Cx) proteins which are capable of forming gap junction channels. Maglione et al. could show in 2010 that the functional oligodendrocyte coupling via gap junctions depends on connexin 47. Additionally, they could prove that the majority of these coupled cells belong to the oligodendrocytic lineage, as they were CNPase-positive oligodendrocytes, NG2- or Olig2-positive precursor cells.

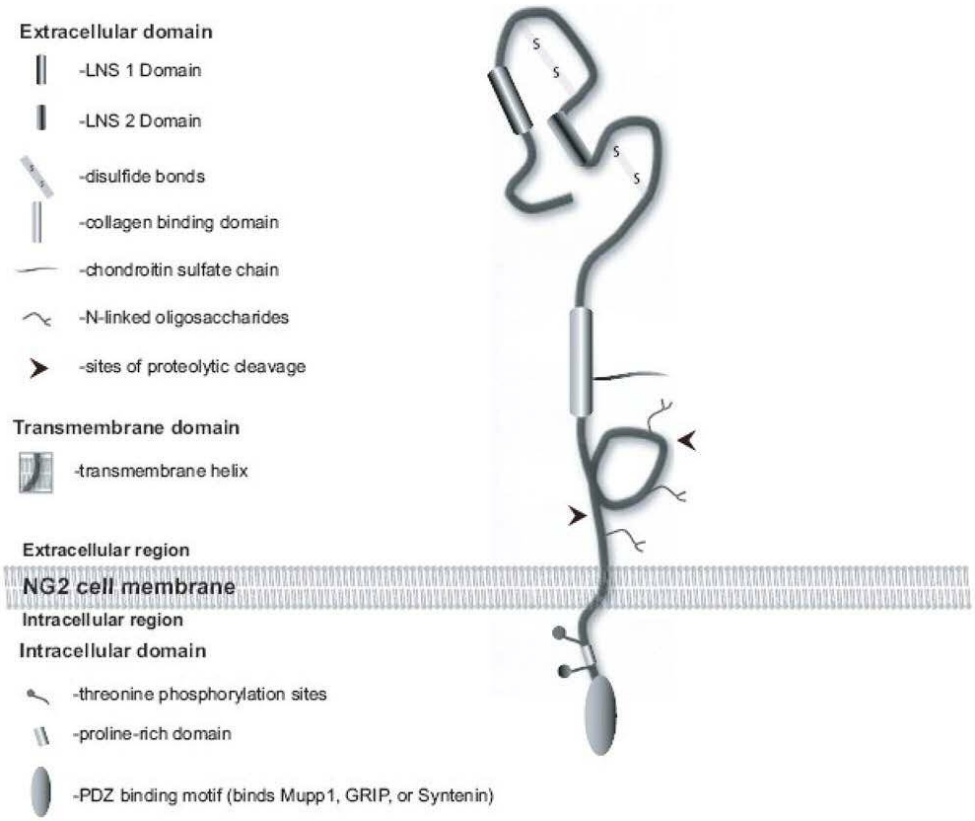

Fig. 8: Schematic diagram of the NG2 protein (taken from Trotter et al. 2010, originally modified from Stallcup and Huang, 2008)

A single gene with multiple exons encodes for the NG2 protein of 330 kDa. NG2 consists of 2327 amino acids (aa). The protein has got a very large extracellular domain, which contains two Laminin G/Neurexin/Sex-hormone binding-globulin (LNS) domains near the NH2 terminus (Fig. 8). Because of these domains, NG2 can be considered as a member of the family of neurexins. Inside the large extracellular domain of NG2, close to the single transmembrane domain, cleaving sites exist, where different proteases are able to affect *in vitro* and *in vivo* (Nishiyama et al., 2009). Additionally, chondroitin sulphate glycosaminoglycan (GAG) chains are linked to the extracellular domain (Nishiyama et al., 2009), which make NG2 to a part-time proteoglycan, as the amount of GAG chains carried by the core protein varies with cell type and developmental stage (Schneider et al., 2001). The short single transmembrane helix consists of 25 aa and is followed by an intracellular part of the protein which contains a PDZ (Postsynaptic density protein-95, Discs-large, Zonula occludens-1) binding motif at the C terminus. The form of the NG2 protein enables it to interact with extracellular and intracellular binding partners (Fig. 9). For instance PDGFα receptor and PDGF-AA ligand (Goretzki et al., 1999; Nishiyama et al., 1996) are extracellular binding partners, while the scaffolding protein known as glutamate receptor interaction partner (GRIP) and the Multi-PDZ domain protein 1 (Mupp1) bind on the intracellular PDZ motif (Stegmuller et al., 2003; Barritt et al., 2000).

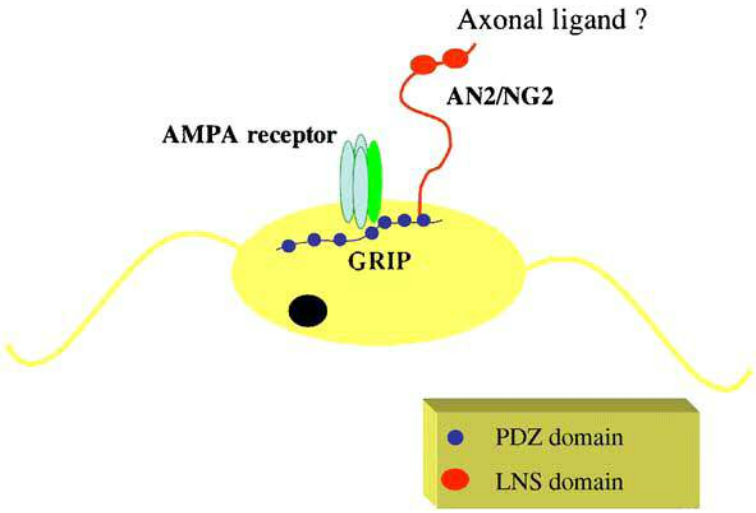

Fig. 9: Complex formed between NG2, GRIP and AMPA receptors in immature glia cells (taken from Trotter et al. 2005)

In 2008 Chatterjee et al. could additionally show that a subset of NG2 proteins is able to bind on the PDZ motif with syntenin-1 in a molecular complex required for cellular motility. This binding seems to be necessary for normal migration.

1.5.2. NG2-EYFP mouse line

In 2005, Karram et al. developed a mouse line in order to facilitate studies on the function of NG2+ cells and to characterize these cells *in situ*. For this purpose they generated an enhanced yellow fluorescent protein (EYFP) ‘‘knock-in mouse’’ (Karram et al., 2008). Therefore, they devised a special knock-in strategy in order to label NG2-expressing cells with EYFP. With the designed targeting vector they inserted the EYFP cDNA into exon 1 of the NG2 gene by homologous recombination in mouse embryonic stem cells. Male animals from the F1 generation, which carried the modified NG2 gene, were bred with “Cre-deleter” (Ella-Cre, Lakso et al., 1996) transgenic female mice to excise the Neo^R gene from the F2 generation. EYFP-expressing cells could be then observed in the F2 generation. From this point on, heterozygous mice which expressed the NG2 protein in all regions and at all ages studied, were inbred in order to obtain the NG2 null mutants. On cell levels wild-type (+/+) and heterozygous (+/-) mice express the NG2 protein, while the homozygous mouse (-/-) lacks the NG2 protein. Vice-versa NG2-EYFP heterozygous and homozygous mice express EYFP, while the wild-type animals lack any expression of EYFP.

1.6. Conditional mouse mutants

In order to gain data that can be relevant to human diseases, appropriate animal models are needed. These models have to show a high similarity to humans on the level of physiology, anatomy and the genes. Over the last years, mice evolved to the most used animal model in research, not only because of their remarkable genetic similarity to humans, combined with a great convenience, but as well because these animals are small and inexpensive to maintain. The short life span and rapid reproductive rate of mice make it possible to study disease processes in many individuals throughout their life cycle.

With the event of genetic engineering, we are now able to create custom mouse models to define gene functions and to relate them to functions in humans (Van der Weyden et al., 2002). By generating a mouse model, mouse geneticists can eliminate (knock-out), overexpress or add (transgenic or knock-in) genes in the whole animal or in a specific chosen tissue/cell population. Inbred strains of mice also provide the opportunity to study a disease trait in a defined genetic background. On the other side, generating mammalian models requires much time and large populations of the animals. The genetic modification of a mouse line is generated by the so called “gene targeting” technique, which consists of the introduction of specific mutations into a gene of interest by homologous recombination using embryonic stem cell technology. After several years of investigation, it became clear that the Cre recombinase of the P1 bacteriophage efficiently catalyses recombination between two of its consensus 34 bp DNA recognition sites (*loxP* sites) in any cellular environment and on any kind of DNA (Nagy, 2000). This gave geneticists the possibility to develop conditional knock-outs, which lack a particular gene only in a specific tissue or after a specific stage of development.

Generation of conditional mutant mice by using the Cre/loxP recombination system

As with transgenic mice, compensatory mechanisms during development may be able to compensate the gene mutation and mask the phenotype of the animals. Another possibility occurs that the widespread deletion of a single gene may be lethal. This problem can be solved by the production of a conditional mutant mouse line, which harbours the knock-out only in specific tissues and allows other tissues to develop normally to support life of the animals. This means that a conditional mutant mouse is a mouse where the mutation of the animal is based on a tissue-specific inactivation of the gene of interest. This can be achieved by means of the specific Cre recombinase. This recombinase of the P1 bacteriophage is a member of the integrase family of site specific recombinases. It catalyses the recombination between two *loxP* sites. In Fig. 10, a *loxP* site is shown, which consists of three different

parts. First, one core spacer with a sequence of 8 bp and two 13 bp palindromic sequences is needed. These sequences flank the core spacer on both sides (a). The 8 bp of the core spacer is ordered in an asymmetric way and by this defines the orientation to the *loxP* site. If both *loxP* sequences are arranged in the same direction, the result will be designed in an excision of the intervening DNA by Cre recombinase and one intact *loxP* site will be left (Fig. 10.b) inside the string. The orientation of the two *loxP* sites in opposite directions leads to an inversion of the DNA sequence (Fig. 10.c).

The advantage, why this system can be used, is that the *loxP* sites do not occur naturally in any mammalian genome. If a specific gene should be integrated or excised, a targeting construct with both flanking *loxP* sites and the specific gene has to be created. Afterwards this construct is integrated in the embryonic stem cell genome. It is important that those *loxP* sites do not disturb any coding regions, regulatory elements or proper splicing (Nagy and Mar, 2001).

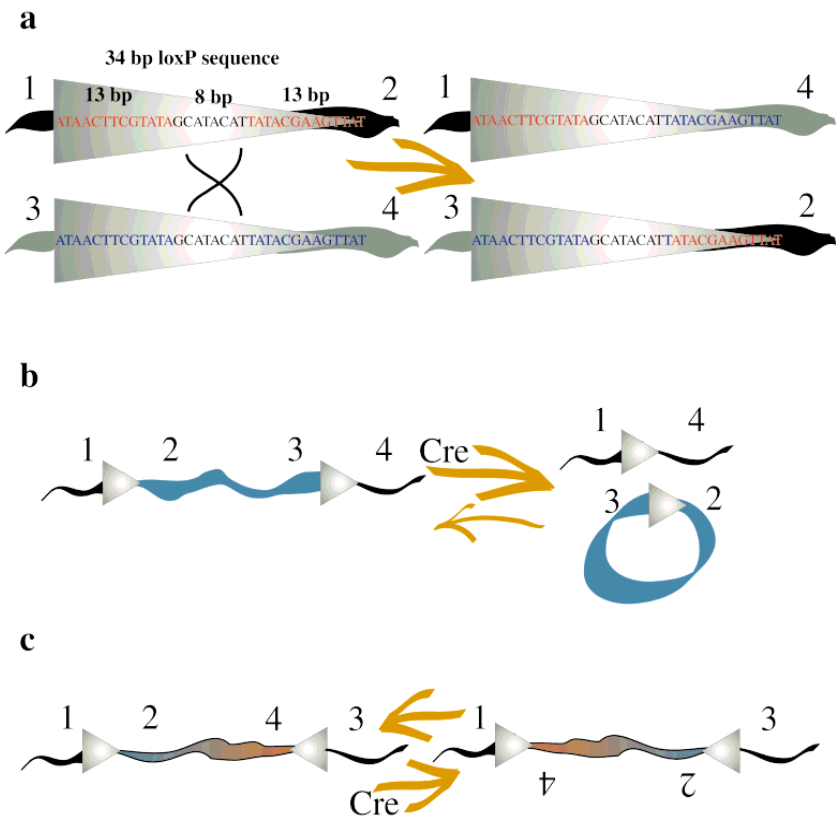

Fig. 10: Cre/*loxP* system. (**a**) Close-up of Cre recombinase-mediated recombination between two 34bp *loxP* sites. Schematics of excision vs. integration (**b**) and inversion (**c**) (modified from Nagy, 2000)

To generate a conditional knock-out mouse line, two *loxP* sites have to be inserted in 5' and 3' direction of the target gene first by a homologous recombination in the genome of embry-

onic stem cells. Mice, which will later develop from these stem cells, express the floxed (flanked by *loxP* sites) gene in a wild-type manner.

The development of selectable markers like e.g. a neomycin-resistance cassette introduced to modified loci, which later can be removed from the targeted alleles, can be used for the identification of targeted alleles. After a favoured insertion is achieved, the marker is deleted by the Cre-recombinase. After this, the floxed gene should be functionally equivalent to the wild type gene and the homozygous mice should be normal. The excision of the floxed gene out of the genome depends on the expression of Cre recombinase. Only if Cre is expressed, it can remove the *loxP*-flanked DNA sequence from the altered alleles. This incident produces a so-called Cre-excision-dependent knock-out mouse. By using a cell type-specific Cre transgene the lineage will be deficient for expressing Cre. In addition to this generation of a floxed mouse line, a second mouse line is needed. This mouse line should express the Cre recombinase under the control of a selected tissue- or cell-specific promoter. The crossing of both lines leads to spatial control of the desired gene alteration in the offspring animals.

CB1 receptor conditional mouse lines

Over the past years, several different transgenic mouse lines were generated in our working group in order to explore the ECS. Since in 1999, Marsicano and Lutz showed that CB1-expressing cells can be divided into different neuronal subpopulations. Since then, it became the major focus of our investigation to have a closer look into the involvement of CB1 on these subpopulations in different physiological and pathological processes as well as in cannabinoids` pharmacological actions. In order to gain more insight about these patterns, several conditional mutant mouse lines were generated (Marsicano et al., 2003; Monory et al., 2006; Maresz et al., 2007; Monory et al., 2007). The first established mouse model was the CB1-null ($CB1^{-/-}$) mutant line, which has no CB1 receptor in any cells (Marsicano et al., 2002). The investigation of this CB1-null mutant line brought new insights into the functions of the ECS. Next, the so called $CB1^{f/f;CAMKII\alpha\text{-}Cre}$ was created in order to delete the CB1 receptor in all principal neurons of the forebrain but not in cortical GABAergic interneurons and not in cerebellar neurons. This mouse line lacks CB1 expression in all projecting forebrain neurons like cortical glutamatergic neurons, thalamic projecting neurons, or striatal medium spiny neurons (MSNs). $CB1^{f/f;CAMKII\alpha\text{-}Cre}$ were generated by crossing $CB1^{f/f}$, a mouse line where the CB1receptor coding region is flanked by two *loxP* sites (Marsicano et al., 2003) with a mouse line, which expresses Cre recombinase under the control of the regulatory sequences of the Ca^{2+}/calmodulin-dependent kinase IIα gene (Casanova et al., 2001). To obtain a more selective deletion of the CB1 receptor in glutamatergic cortical neurons, $CB1^{f/f}$ mice were crossed

with NEX-Cre transgenic mice, which express Cre recombinase under the control of the regulatory sequences of the neuronal differentiation factor NEX (Kleppisch et al., 2003), so that the double mutants $CB1^{f/f;NEX\text{-}Cre}$ were received. The helix-loop-helix transcription factor NEX is a marker of embryonic neuronal progenitors, which will develop into mature glutamatergic cortical neurons (Wu et al., 2005). In the $CB1^{f/f;NEX\text{-}Cre}$ mice, CB1 expression is absent in the majority of cortical glutamatergic neurons, including hippocampus, neocortex and amygdala, whereas GABAergic interneurons and other subcortical neurons express normal levels of the receptor. Next, animals of the floxed $CB1^{f/f}$ mouse line were crossed with Dlx5/6-Cre animals. $CB1^{f/f;dlx5/6\text{-}Cre}$ mice lack expression of CB1 in practically all the GABAergic neurons of the forebrain. With the help of these conditional mutant mouse lines it should be possible to differentiate between the functions of CB1 on GABAergic and glutamatergic neurons (Monory et al. 2006). Shortly afterwards the $CB1^{f/f;D1\text{-}Cre}$ mouse line was generated. For this line, $CB1^{f/f}$ mice were crossed with a transgenic mouse line where Cre recombinase is expressed under the control of the regulatory sequences of dopamine receptor D1 (D1-Cre), in order to obtain a specific deletion of CB1 in striatal neurons (Lemberger et al., 2007; Mantamadiotis et al., 2002). In $CB1^{f/f;D1\text{-}Cre}$ mice, the great majority of MSNs of caudate putamen (striatum) does not show CB1 receptor expression. However, the dopamine D1 receptor is expressed not only in striatal neurons, but also in other brain regions. The Cre-mediated recombination under the control of D1 regulatory sequences takes also place in the layer VI of the neocortex (Lemberger et al., 2007; Mantamadiotis et al., 2002). In this region, CB1 mRNA is present both at high levels in GABAergic interneurons (Marsicano and Lutz, 1999) and at low levels in principal glutamatergic neurons (Monory et al., 2006). However, as D1 receptor is not expressed in GABAergic interneurons and the Cre recombinase is expressed under the control of the regulatory sequences of dopamine receptor D1, Cre-mediated recombination can only take place in the projecting glutamatergic neurons. Our group has published the first report about the $CB1^{f/f;D1\text{-}Cre}$ mice (Monory et al., 2007). As described in this publication, CB1 receptor is absent in the great majority of striatal non-D2 expressing neurons of $CB1^{f/f;D1\text{-}Cre}$ mutant mice. It is important to mention that non-D1 and non-D2 neurons in the caudate putamen keep their expression of CB1 receptor in the mutant mice. These neurons very likely belong to the population of striatal GABAergic interneurons, which were shown to contain CB1 mRNA (Hohmann and Herkenham, 2000) as well.

The transgenic $CB1^{f/f;D1\text{-}Cre}$ mouse line was used in this study in order to test its behaviour in different models. Additionally, gene expression changes in the striatum of $CB1^{f/f;D1\text{-}Cre}$ mice were evaluated by GeneChip Mouse Genome 430A 2.0 Array from Affymetrix after a conditioned place preference experiment with repeated nicotine injections.

1.7. Behavioural analyses

Behavioural analyses, as they are known today, started already more than 100 years ago with Darwin´s systematic studies of animal behaviour. With the help of behavioural analyses it was, and still is, possible to study animals in their natural environment without an influence of the observer. The central questions are which internal or external factors impact specific reactions of the animals and how behaviour is controlled. But with time, behavioural genetics outgrew the observation of species and strain differences. With the advent of behavioural genetics and behavioural neuroscience, behavioural analysis became even more important. The knowledge that genes and environment are both essential and interdependent determinants of behavioural responses, made behavioural genetics focus even more on the role of genes on behaviour (De Sousa et al., 2006). Today, research focuses on mechanisms underlying observed behaviours and the interaction of genetic, anatomical, physiological, biochemical and environmental influences. Every new genetically modified mouse line, independent whether it is transgenic, knock-out or knock-in, needs special observing of their behaviour in order to reveal whether there are changes in behaviour compared to wild-type mice. To evaluate behaviour, specific tests were developed. Over the last years, different laboratories and scientists worked out many various methods for behavioural phenotyping of transgenic and knock-out mice (Crawley, 1999).
In this study the transgenic mouse lines, $CB1^{f/f;D1\text{-}Cre}$ and NG2-EYFP were tested in different models of behavioural tasks to find out which differences appear in these conditional mutant mice compared to their wild-type littermates. Additionally, the $CB1^{f/f;D1\text{-}Cre}$ mouse line was compared to other conditional mouse lines of our laboratory in order to gain more insight, which effect the lack of CB1 receptor on D1-expressing MSNs in the area of striatum has on certain behaviours and how important the background of the animals is for that behaviour.

Characterization of basic functions

Before one starts different experiments to test certain paradigms of rodent's behaviour in special models, the first step in behavioural phenotyping is to detect, whether the animals have general abnormalities or deficits which will obviously limit the planned experiment. A mouse which shows deficits in locomotion will not be able to perform in a lot of behavioural challenges. Neither will a deaf animal react to tones in auditory tasks. Therefore, it is obligatory that the phenotype of a mouse line is first checked in its general home cage behaviour.

Measurement of body weight

The measurement of general body weight is usually the common first step before an animal behavioural experiment. Animals which are chosen for such an experiment are weighed before the start of tests in order to avoid locomotion discrepancies by weight differences. For the measurement each mouse is put in a beaker and weighed with a standard laboratory balance.

Acoustic startle response

The acoustic startle response test provides a good measure of gross hearing ability of the animals (Crawley, 1999). Acoustic startle is a reflex that can be seen in most mammals. It is a yes-or-no-response measured by a human observer. After presenting a loud tone, the observer checks the reaction of the animals based on their body flinch and vocalization. With this paradigm, it can be evaluated, whether the animals show deficits in the hearing ability. A mouse line with auditory disabilities should not be tested in auditory tasks. Additionally, it is obvious that the behaviour of a deaf mouse might be altered in most behavioural experiments, due to changes in anxiety.

Models of locomotion and motor functions

At the beginning of a battery of behavioural analyses one should be aware that it is important to perform various tests of motor functions before starting with more complex paradigms, e.g. a cognition task. Obviously, an impairment of motor functions makes it impossible to observe the animals in more complex or difficult tasks, solely because they cannot walk or move. An impairment of motor functions can identify meaningful interpretations of the behavioural phenotype of a tested mouse (Crawley, 1999). Models of emotionality and cognition have to rely on the movement of the animal providing an indirect measure. Therefore motor functions have to be the primary interest in any behavioural test.

Open field test

The most standardized measurement of locomotion and motor functions is the activity of mice in the open field within a predetermined time period. In the open field test the baseline levels of locomotor activity in transgenic and knock-out mice can be compared to their wild-type littermate controls. As already mentioned before, the striatum, with its high density of CB1 and D1 receptors takes part in control of locomotion. Cannabinoid receptor agonists have been reported to impair the distance moved in the open field. Also antipsychotics such as haloperidol, a potent dopamine D2 receptor antagonist, impair the behaviour in this mod-

el. This suggests that both cannabinoid receptors and dopamine receptors in the brain are involved in locomotion (Nagai et al., 2006).

In the open field, motor activity , horizontal and/or vertical movement can be measured in a test environment. For the detection, a special automated system is necessary (video analysis or infrared beams) (De Sousa et al. 2006). Preferentially the animals must not be trained in this model and should be used naively (Crusio, 2001; Crawley, 1999).

Grip strength test

Rodents exhibit a gripping reflex which can be measured by the grip strength test (van Riezen and Boersma, 1969). By measuring the grip strength, neuromuscular function and muscular strength can be assessed. In this task, the peak amount of force that is required to make a mouse release its grip is determined. Forelimb grip strength is measured as tension force using a grip strength meter (GSM). It measures the maximal muscle strength of fore-limbs and combined forelimbs and hind limbs as a primary phenotype screen.

Rotarod test

The rotarod apparatus consists of a rotating beam, of approximately three centimetres in diameter, where five mice can be placed at the same time, separated by plastic walls. The ability of the mice to walk on this rotating beam or their performance on the rotarod describes a measure of motor coordination, balance and motor learning. The motor learning can be estimated by repeated daily testing on the rotarod. Reduction in latency to fall down, normally over repeated testing sessions, provides a measure of cerebellar learning (Crawley, 1999). The striatum with his high density of CB1 and D1 receptors takes part both in locomotion and in motor learning. With this model it is possible to review whether a mouse line is able to per-form on a rotating beam. For instance if there is a mutation that affects cerebellar function, mice will perform differently than wild-type animals. As described previously (Bilkei-Gorzo et al., 2005; Serradj et al., 2006), the performance in this task can be age-related as well. But not only age or mutations affect the behaviour on the rotarod. The genetic background of the mutant animals is also important information as different strains have different performance abilities on the rod.

Models of emotion

In order to work with the models of emotion it is important to keep in mind that no one is able to compare or equate emotional behaviour of animals with the emotions humans know and feel. It is impossible to say whether a mouse feels afraid, anxious or depressed, because humans cannot interpret the feeling of an animal. Feelings are always based on subjective emotional experiences, which exist in the mind and body of the individual (Crawley 1999). However, it is possible to observe behavioural and physiological responses to different stimuli and events which can be interpreted as an indirect measure of emotional responses.

Elevated plus maze test

The elevated plus maze (EPM) is a test of rodent anxiety-related behaviour. This behaviour originates from the inherent conflict of an "approach and avoid" situation. More precisely, this model is based on a natural conflict of the animals between the tendency to explore a novel environment and the aversion of a brightly lit and open area. The EPM consists of two open and two closed arms with plastic walls. The platform of the EPM, where the animals walk, is usually around one meter in height. This height and the open space of the open arms confront the animals with another anxiety-producing factor (Carobrez and Bertoglio, 2005). The mice can decide to stay in the darker enclosed arms or to explore the maze and step in the brighter open arms. An increase in open arms exploration is an index of reduced anxiety-like behaviour. The genetic modification in a mouse line can cause differences in the behaviour, shown in this paradigm, compared to wild-type littermates. But not only genetic alterations change the behaviour. Drugs which influence the brain regions responsible to anxiety can be used to change the natural behaviour of mice as well. For example, it is known that Δ^9-THC can cause anxiogenic or anxiolytic effects on behaviour on the EPM depending on the used dose (Rubino et al., 2007; Patel and Hillard, 2006).

Light-dark test

In the early 1980´s, J. Crawley and colleagues invented the light-dark-test (LD), which is similar to the EPM and takes advantage of the natural conflict of the animals between the tendency to explore unknown areas and the aversion of a brightly lit open environment. Crawley and colleagues combined the concepts of the open field test with the social interaction test and created this model, which is now commonly used in behavioural testing, because of its simple arrangement (Crawley, 1999). A usually used LD box consists of a white open area and a closed, dark compartment. The level of exploration between these different compartments relates to the basal level of anxiety in novel situations. Therefore in the LD, drug-induced increase in activity (without an increase in spontaneous locomotion) in the light

part of a two-compartment box, in which a large white compartment is illuminated and a small black compartment is darkened, is interpreted as an index of anxiolytic activity (Bourin and Hascoet, 2003).

Forced swim test

The forced swim test (FST) is the most widely used test for assessment of depression-like behaviour in animal models (Cryan and Holmes, 2005). The test is commonly used to measure the effect of antidepressant drugs on behaviour, but can reveal a basal depression-like phenotype difference between genotypes as well. It is based on the fact that mice swim in the water to seek an escape route (Crawley, 1999). Originally, the FST was developed for rats (Porsolt et al., 1977), but was shortly afterwards translated to mice as well (Porsolt et al., 1978). The animal is usually forced to swim within a round glass beaker for six minutes, where the mouse is unable to touch the bottom with the feet or tail. Confronted with such a situation, the mouse first tries to escape and shows a “struggling” behaviour, which means that it breaks the water surface with its front paws and tries to climb out of the water. Over the swim period the animal “realizes” that swimming behaviour leads to no escape and it reduces its swimming movements towards more and more immobile “floating” behaviour, where the animal shows just the necessary paw movements which are needed to stay afloat. Animals which float more over the test period can be said to have a stronger depression-like phenotype.

Models of recognition memory and learning

Novel object recognition task

The novel object recognition task (NORT) is used to measure non spatial memory in rodent models (Ennaceur and Delacour, 1988). This test is performed in a common open field box where on one side objects will be placed. The NORT is based upon the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object more reflects learning and recognition memory, if the animals recognize the old one as familiar. This paradigm combines a general exploration test with a visual recognition memory paradigm. Therefore, it can be used to measure object exploration and object recognition in the animals. Additionally, this paradigm is able to give information on short-term or long term memory and the promnesic or the amnesic effect of a drug.

Morris water maze task

The Morris water maze task (MWM) is one of the most popular tasks in behavioural neuroscience. Originally, this task was established by Richard Morris (1984) for rats, but later modified for the testing of mice as well. In its most basic form, the water maze assesses spatial learning and memory. It is a spatial navigation task, where the animals have to swim in order to find a hidden platform, which represents their only possibility to escape the water. The task is based on the principle that mice are highly motivated to escape from a water environment by the quickest and direct route (Crawley, 1999) by using the help of visual landmarks outside the pool. Performance in the MWM is acutely sensitive to manipulations of changes in the hippocampus, but age and genetic background of the animals can influence the performance as well (Wahlsten et al., 2003).

Sociability test

In humans, deficits in social interaction are important early markers for autism and related neurodevelopmental disorders with strong genetic components. Generally, mice are a highly social species. Therefore, their interest in social contacts is used to evaluate differences in social behaviours, which can be seen as a model for neurodevelopmental disorders in humans.

The modified sociability test is based on the protocol of Moy et al. (2004). They developed a new procedure to assess sociability and the preference for social novelty in mice. With a three compartment test chamber they provided a method either to measure the time spent with a novel mouse (conspecific) or to test for the preference for a novel vs. a familiar conspecific. In order to quantify sociability, the test mouse is first habituated to the central compartment. Afterwards it is scored on measures of exploration in the central compartment, the side compartment which contains an unfamiliar mouse in a wire cage, and the second, empty side compartment. The preference for social novelty is tested two hours after the sociability test. By presenting the test mouse the possibility to choose between the first, now-familiar, mouse in one side chamber, and a second unfamiliar interaction partner in the other side chamber, social novelty can be measured by the difference in time spent with the familiar and the new interaction partner.

Resident-intruder test

The paradigm of the resident-intruder test allows the evaluation of social exploration and aggressive behaviour in rodents (Goyens & Noirot, 1975). Actually, this test is a modification of the standard opponent test, which takes place in the home cage of a test (resident) mouse (Crawley, 1999). Usually, the male intruder in this paradigm prompts the territorial attacks of another male, but resident, test mouse in its own home cage. The duration, percentage of

time and number of fights can be evaluated and provide a measurement for aggressive behaviour. A physical struggle between the interaction partners which is initiated by an attack of the resident mouse towards the intruder mouse is scored as a fighting period. Different ages and housing circumstances provide a possibility to modify the interaction between the animals. By placing a novel, younger, group housed intruder mouse into the home cage of an older single-housed animal, the number of interactions induced by the intruder can be decreased.

Models of addiction

Drug-induced reinforcement can be assessed directly in mice. To this end, different methods can be used, including operant or non-operant self-administration, classical conditioning-based paradigms such as conditioned place preference or sign tracking, facilitation of intracranial electric self-stimulation, or drug-induced memory enhancement (Sanchis-Segura and Spanagel, 2006). Drug addiction is seen as a chronic relapsing brain disorder characterized by neurobiological changes leading to compulsive drug seeking and drug taking despite serious negative consequences, and by the loss of control over drug use (Camí and Farré, 2003). Addiction integrates intricate different behavioural and neurobiological processes. All the drugs of abuse, e.g. nicotine, Δ^9-THC or cocaine, produce reinforcing effects accountable for the initiation of the addictive disorder (Maldonado et al., 2006).

Conditioned place preference test

Conditioned place preference (CPP) is one of the most popular experimental protocols used to measure drug reward or subjective effects of a drug in laboratory animals. This model is based on modern and traditional theoretical formulations of Pavlovian conditioning and it seems to reflect a preference for a context. This reflection relies on the association between the surrounding and a drug stimulus (Bardo and Bevins, 2000). First a drug is injected and the subject is placed in a test chamber with distinctive environmental cues. This procedure is repeated for several days. During this conditioning phase the mouse develops an association between the subjective state produced by the drug (e.g. reward comparable to mood elevation and euphoria in humans) and the environmental cues which are present during the drug state. When the subject is tested after the conditioning phase in the same apparatus that contains the drug-related environmental cues in one compartment and neutral, non-drug associated cues in another, it voluntarily moves toward the compartment containing the drug-related cues. This learned association between environmental stimuli and a positive drug

effect provides the basis for CPP experiments. The comparison of the behaviour of the tested animals in the pre-test before and after the conditioning phase, the so-called “increase of the time spent in a drug-associated compartment”, can be considered as a measure of conditioned preference.

Conditioned place aversion test

The conditioned place aversion (CPA) test uses the same apparatus and procedures as the CPP test. Again, one compartment is associated with drug injections, whereas the other is coupled to neutral, non-drug-associated cues and vehicle administration. The experiment starts with a pre-test where the animal is allowed to explore the whole apparatus without any injections. Afterwards, a conditioning phase of changing drug/vehicle administration follows, and the experiment ends with an injection-free post-test, in which the animal will be allowed to move freely across all compartments again under the same conditions as on the pre-test day. The decrease in the time spent in the drug-associated compartment is considered as a measure of conditioned place aversion (Jackson et al., 2009). The aversion in the tested animals, which will be caused in this model, can be coupled to negative experience or effects, triggered by a distinct drug itself or by withdrawal effects, released by blockage of already chronic drug effects. If one is working with withdrawal effects, it is important that the animals are in a chronic addicted state before the experiment starts.

Model of excitotoxic seizures

Excessive excitatory activity leads to neuronal damage and death, induced by a mechanism known as excitotoxicity (Ben-Ari and Cossart, 2000). This excitotoxicity is a pathological process where nerve cells are damaged and killed by an excessive stimulation by the neurotransmitter glutamate. The spreading of uncontrolled glutamatergic neurotransmission by an overactivation of the glutamate receptors such as the NMDA receptor and AMPA receptor causes seizures. In animal experiments this state can be caused by injections of kainic acid, a natural marine acid present in some seaweeds, which acts through specific kainate receptors and is neuroexcitotoxic and epileptogenic.

Excitotoxins such as kainic acid are able to bind to kainate receptors, as well as a pathologically high level of glutamate, can cause excitotoxicity by opening calcium channels which leads to a high level of calcium ions in the cell. This Ca^{2+} influx activates a number of enzymes, which are able to damage cell structures such as components of the cytoskeleton, the membrane and the DNA.

2. Aim of study

2.1. CB1 receptor conditional mutant mice and the endocannabinoid system

The ECS is involved in many physiological processes, including the regulation of locomotion (Chaouloff et al., 2011; Sañudo-Peña et al., 2000), emotion (Moreira and Lutz, 2008; Moreira et al., 2008), addiction (Maldonado et al. 2006) and memory processing (Marsicano and Lutz, 2006). However, it has not yet been clarified in details which neuronal mechanisms and circuits are involved in these processes and how they are precisely affected by the ECS.

With the help of conditional mutant mouse lines, it is possible to explore the functions of the ECS on specific cell populations. In our laboratory, several different transgenic mouse lines have been generated during the last years in order to enable such investigations. The generation of the $CB1^{f/f;D1\text{-}Cre}$ mouse line, where the great majority of medium spiny neurons of the caudate putamen (striatum) does not express the CB1 receptor anymore, provided an animal model by which the interaction of the ECS and the dopaminergic system can be evaluated. Dopamine D1 receptor is expressed not only in striatal neurons, but also in other brain regions. The Cre recombinase-mediated gene inactivation under the control of regulatory sequences of the D1 receptor gene takes also place in layer VI of the neocortex (Lemberger et al., 2007; Mantamadiotis et al., 2002). The main aim of the present study was to explore the functions of the CB1 receptor in the D1 dopamine receptor-expressing neuronal population in the brain with the help of the conditional $CB1^{f/f;D1\text{-}Cre}$ mouse line. D1 dopamine receptor expressing ("dopaminoceptive") neurons are known to be involved in physiological functions such as locomotion, reward/addiction and emotion. Based on this knowledge, the $CB1^{f/f;D1\text{-}Cre}$ mutant mice should be tested for possible alterations in different behavioural paradigms such as the open field, the rotarod, the conditioned place preference test, the elevated plus maze, the light-dark test, and the novel object recognition test. As the addiction model and the models of recognition memory and learning are of particularly interest, these paradigms will be tested with other CB1 receptor mutant lines to understand the exact participation of the ECS in these processes.

Therefore, I additionally aim at working with the CB1-null mutant mice ($CB1^{-/-}$), which lack the CB1 receptor in all cells (Marsicano et al., 2002). Also, I use the mutant mice $CB1^{f/f;NEX\text{-}Cre}$, obtained by crossing $CB1^{f/f}$ mice with NEX-Cre mice (Kleppisch et al., 2003; Wu et al., 2005). In this mutant line, CB1 receptor expression is absent in most, if not all, cortical glutamatergic neurons, including hippocampus, neocortex and amygdala, whereas GABAergic interneurons and other subcortical neurons express normal levels of the receptor. Additionally, $CB1^{f/f;dlx5/6\text{-}Cre}$ mice were generated in our laboratory. These animals lack the expression of

CB1 receptor in virtually all GABAergic neurons of the forebrain. With the help of these two mouse lines, it should be possible to differentiate between the functions of CB1 receptor on GABAergic and glutamatergic neurons (Monory et al., 2006) concerning certain behaviour. Additionally, I compare the $CB1^{f/f;D1\text{-}Cre}$ mouse line in different models of recognition memory with animals of the $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ mouse line. Furthermore, I test the $CB1^{f/f;D1\text{-}Cre}$ mouse line in models of addiction and compare the animals with the $CB1^{-/-}$ mouse line. All these experiments are done in order to explore the ECS on specific neuronal populations.

To investigate the general influence of the genetic background of mutant mouse lines (C57BL/6N vs. C57BL/6J) on addiction behaviour, I aim at evaluating the behaviour of $CB1^{f/f;D1\text{-}Cre}$ animals, bred on C57BL/6N background, and J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mice, bred on C57BL/6J background, as well as animals of CB1 null-mutant mouse lines on C57BL/6N ($CB1^{-/-}$) and C57BL/6J background (J-$CB1^{-/-}$) in models of addiction in order to answer the question whether the effect of different backgrounds might influence the addictive effects of nicotine and cocaine.

Additionally, I wish to evaluate gene expression changes in the striatum of $CB1^{f/f;D1\text{-}Cre}$ mice by using a GeneChip Mouse Genome 430A 2.0 Array from Affymetrix after a conditioned place preference experiment with repeated nicotine injections. Obtained genes are validated by qPCR experiments. These results are compared with the results gained from respective treated animals of the J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mouse line. Not only the effect of genes on the behaviour of the animals, but additionally the effect of a particular behavioural paradigm combined with drug treatment on the gene expression will be evaluated in the $CB1^{f/f;D1\text{-}Cre}$ mice with different genetic background.

2.2. Behavioural characterization of NG2-EYFP mouse line

The human brain contains approximately the same amount of neuroglia cells and neurons. Glial cells are non-neuronal cells in the brain which help to maintain homeostasis, form myelin and provide structural and metabolic support, and protection for neurons. Additionally, they have a central role in promoting the formation of synapses and modulation of synaptic activity. But it is still not clear how glial cells participate in the formation of memory processes and behaviour. Thus, the general aim of the second part of the present study is to explore the function of the NG2 protein expressed in oligodendrocytes. It was shown that NG2+ cells make contacts with glutamatergic and GABAergic cells via direct neuron-glia synapses (Lin and Bergles, 2004; Bergles et al. 2000). The release of glutamate or GABA from neurons at these synapses causes a rapid activation of glial AMPA or $GABA_A$ receptors (Lin et al., 2005;

Bergles et al. 2000). Stegmuller et al. could show in 2003 that the NG2 protein assembles with the glial AMPA receptors via GRIP. Thus, it is possible that NG2 could be a part of these neuron-glia synapses. Furthermore, the PDZ motif of the NG2 protein binding to syntenin-1 seems to be necessary for normal migration rates of oligodendrocyte precursor cells (Chatterjee et al., 2008). I aim at exploring the NG2 function in the intact animal by using the NG2-EYFP knock-out mouse line (Karram et al., 2008) and performing a series of behaviour analyses.

3. Materials

3.1 Chemicals

Substance	Company
Ammonium acetate (NH_4OAc)	Merck, Darmstadt (Germany)
Chloroform	Roth, Karlsruhe (Germany)
Dextran sulphate	Roche Molecular Diagnostics, Basel (Switzerland)
Diethylpyrocarbonat (DEPC)	Roth, Karlsruhe (Germany)
Dimethyl sulfoxide (DMSO)	Sigma Chemical Co, St. Luis (USA)
Dithiothreitol (DTT)	Merck, Darmstadt (Germany)
Ethanol	Roth, Karlsruhe (Germany)
Ethidium bromide	Merck, Darmstadt (Germany)
Ethylenediaminetetraacetic acid (EDTA)	Roche Molecular Diagnostics, Basel (Switzerland)
Formamide	Roche Molecular Diagnostics, Basel (Switzerland)
Hydrogen peroxide (H_2O_2)	Merck, Darmstadt (Germany)
Isofluran	Abbott GmbH, Wiesbaden (Germany)
Isopropanol	Roth, Karlsruhe (Germany)
Magnesium chloride ($MgCl_2$)	Merck, Darmstadt (Germany)
Paraformaldehyde (PFA)	Roth, Karlsruhe (Germany)
Phenol	Roth, Karlsruhe (Germany)
Potassium chloride (KCl)	Merck, Darmstadt (Germany)
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck, Darmstadt (Germany)
Ribonucleotides (rATP, rGTP, rCTP)	Roche Molecular Diagnostics, Basel (Switzerland)
RNA ladder	New England Biolabs, Ipswich (USA)
Roche blocking reagent	Roche Molecular Diagnostics, Basel (Switzerland)
Sodium acetate (NaOAc)	Roth, Karlsruhe (Germany)
Sodiumchloride (NaCl)	Merck, Darmstadt (Germany)
Sodiumdodecylsulfate	Sigma Aldrich Chemie GmbH, Munich (Germany)
Sodium hydroxide (NaOH)	Sigma Chemical Co, St. Luis (USA)
Di-Sodium hydrogenphosphate	Merck, Darmstadt (Germany)
Toluidin blue	Serva Feinbiochemica, Heidelberg (Germany)
Transcription buffer 10x	Roche Molecular Diagnostics, Basel (Switzerland)
Triethanolamine	Roth, Karlsruhe (Germany)
Tris base	Roth, Karlsruhe (Germany)
tRNA	Roche Molecular Diagnostics, Basel (Switzerland)

3.2 Drugs and narcotics

Drugs	Company
Atipamezol hydrochloride	Pfizer GmbH, Karlsruhe (Germany)
Cocaine hydrochloride	Sigma Chemical Co, St. Luis (USA)
Fentanyl	Janssen-Cilag GmbH, Neuss (Germany)
Flumazenil	Hikma Pharma GmbH, Nieder-Olm (Germany)
Kainic acid monohydrate	Sigma Chemical Co, St. Luis (USA)
Mecamylamine hydrochloride	Sigma Aldrich Chemie GmbH, Munich (Germany)
Medetomidin hydrochloride	Fort Dodge Veterinär GmbH, Würselen (Germany)
Midazolam	Ratiopharm Gmbh, Ulm (Germany)
Naloxon	Ratiopharm Gmbh, Ulm (Germany)
Novaminsulfon	Ratiopharm Gmbh, Ulm (Germany)
Nicotine 99% GC	Sigma Chemical Co, St. Luis (USA)
Nicotine tartrate salt 98% TLC	Sigma Aldrich Chemie GmbH, Munich (Germany)
Pentobarbital sodium	Sigma Chemical Co, St. Luis (USA)

3.3 Apparatuses

Apparatus	Company
Balance adventurer ARA 520	OHAUS, Giessen (Germany)
Centrifuge 5417C	Eppendorf AG, Hamburg (Germany)
Centrifuge labofuge 400 R	Kendro/Thermo Electron, Langenselbold (Germany)
Conditioning place preference (CPP) boxes	Haustechnik, Universität Mainz (Germany)
Cryostat HM 560	Micro International GmbH, Walldorf (Germany)
DVD-recorder	Sony corporation, (Japan)
Elevated plus maze (EPM)	Haustechnik, Universität Mainz (Germany)
GFC 1083 waterbath	GFC, Burgwedel (Germany)
Grip strength meter (GSM)	TSE, Bad Homburg (Germany)
Incubator	WTB Binder, Tuttlingen (Germany)
Light-dark-boxes (LD)	Haustechnik, Universität Mainz (Germany)
Magnetic stirrer	Fisher Bioblock Scientific, Illkirch Cedex (France)
Morris water maze (MWM)	Haustechnik, Universität Mainz (Germany)
Nanodrop	PEQLAB, Biotechnologie GmbH, Erlangen (Germany)
Open field box	Haustechnik, Universität Mainz (Germany)
Power supply LNG 350-06	Phase GmbH, Lübeck (Germany)
qPCR T7300	Applied Biosystems, Carlsbad (USA)
Rotarod	Ugo Basile, Comerio (Italy)
Shaking incubator	GFL Labortechnik, Wunstorf (Germany)
Thermoblock 5320	Eppendorf AG, Hamburg (Germany)
Thermocycler T 3000	Biometra GmbH, Göttingen (Germany)
Thermomixer	Eppendorf AG, Hamburg (Germany)
TissueLyser II	QIAGEN, Hilden (Germany)
Video camera	Sony corporation, (Japan)
Vortexer VF2	Janke & Kunkel GmbH, Staufen (Germany)

3.4 Kits and other consumable materials

Kits or consumable materials	Company
Ambion MessageAmp™ Premier RNA Amplification Kit	Applied Biosystems, Darmstadt (Germany)
ALZET® osmotic pumps	Charles River, Sulzfeld (Germany)
Falcon tubes	Greiner GmbH, Frickenhausen (Germany)
Plastic counting vials and caps	Zinsser Analytic, Frankfurt (Germany)
QIAquick PCR Purifications Kit	QIAGEN, Hilden (Germany)
QIAGEN RNeasy Mini Kit	QIAGEN, Hilden (Germany)
High capacity cDNA reverse transcription Kit	Applied Biosystems, Carlsbad (USA)
SuperFrostPlus slides	Menzel-Gläser, Braunschweig (Germany)
96-well plates for qPCR	Applied Biosystems, Carlsbad (USA)

3.5 Enzymes

Enzyme	Company
BamHI restriction enzyme	New England Biolabs, Ipswich (USA)
DNaseI (RNase-free)	Roche, Basel (Switzerland)
EcoRI restriction enzyme	New England Biolabs, Ipswich (USA)
T7 RNA polymerase	Promega GmbH, Mannheim (Germany)
T3 RNA polymerase	Promega GmbH, Mannheim (Germany)
Go Taq polymerase	Promega GmbH, Mannheim (Germany)
Proteinase K	Merck, Darmstadt (Germany)
RNasin	Promega GmbH, Mannheim (Germany)
RNaseA	Roth, Karlsruhe (Germany)

3.6 Primers and Probes

Primers are short oligonucleotides of approximately 18-25 bases, which are needed by a DNA-polymerase (e.g. Taq-Polymerase) to start the replication process for the amplification of DNA sequences.

Primer	Sequence
G122	GGC TCA AGA CTC GTC AAA ATC
G123	CGC GCC TGA AGA TAT AGA AGA
G100	CGG CAT GGT GCA AGT TGA ATA
G101	GCG ATC GCT ATT TTC CAT GAG
G50	GCT GTC TCT GGT CCT CTT AAA
G51	GGT GTC ACC TCT GAA AAC AGA
G53	CTC CTG TAT GCC ATA GCT CTT
G54	CCT ACC CGG TAG AAT TAG CTT

TaqMan® Gene Expression Assays are a standardized method for quantitative gene expression. They are built on Applied Biosystems 5' nuclease chemistry and consist of a FAM™ dye-labelled TaqMan® MGB probe (250 nM, final concentration), and two unlabelled PCR primers (900 nM each, final concentration). All components of these assays are quality control tested and formulated into a single 20x mix. These probes are made to work under universal conditions for two-step RT-PCR.

Probes	**Gene**
Mm00479224_m1	Pituitary tumor-transforming gene 1 (*pttg1*)
Mm00476505_m1	Neuronal pentraxin 1 (*nptx1*)
Mm00481140_m1	Neurotensin (*nts*)
Mm00516104_m1	Kruppel-like factor 4 (*klf4*)
Mm00493433_m1	T-box brain gene 1 (*tbr1*)
Mm00443267_m1	Transthyretin (*ttr*)
Mm00456650_m1	Early growth response 2 (*egr2*)
Mm01326464_m1	Neurogenic differentiation 6 (*neurod6*)
Mm01174378_m1	Calmodulin-like 4 (*calml4*)
Mm01212171_s1	Cannabinoid receptor 1 (*cnr1*)
Mm00446956_m1	Glucuronidase, beta (*gusB*)

3.7 Mouse lines

For all experiments, male adult mice between the age of nine weeks and 25 months were used. Animals were separated 7 – 10 days before experiment and kept single housed in a temperature-controlled room (21-22°C; 49-51% humidity) with a 12h-light-dark-cycle (lights on 7:00-19:00) until end of experiments. They had access to food and water *ad libitum*. All experimental procedures were approved by the Committee on Animal Health and Care of the local Government. C57BL/6N or C57BL/6J mice were either taken from our own breeding facility of the institute or purchased from Charles River (Germany). The generation of the mouse line $CB1^{f/f;D1\text{-}Cre}$ was as follows. $CB1^{f/f}$ mice (Marsicano et al., 2003) were crossed with dopamine receptor D1-Cre line (Lemberger et al., 2007; Mantamadiotis et al., 2002), in which the Cre recombinase was placed under the control of the dopamine receptor D1A gene (*Drd1a*) regulatory sequences using transgenesis with modified bacterial artificial chromosomes. The pattern of Cre expression recapitulated the expression pattern of the endogenous *Drd1a* (Lemberger et al., 2007; Mantamadiotis et al., 2002). The $J\text{-}CB1^{f/f;J\text{-}D1\text{-}Cre}$ mouse line only differed in the genetic background, it was backcrossed to C57BL/6J for >10 generations. $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ mice were obtained by crossing $CB1^{f/f}$ animals with NEX-Cre or DLX-Cre mice, respectively (Monory et al., 2006). J-CB1-null mutant mice (called J-

CB1$^{-/-}$) and wild-type littermates (J-CB1$^{+/+}$) on C57BL/6J background from heterozygous breeding were used for the experiments analogue to the published CB1-null mutant mice (called CB1$^{-/-}$) and their respective wild-type littermates (CB1$^{+/+}$) (Marsicano et al., 2002).

NG2-EYFP mice were provided for behavioural experiments by the group of Professor J. Trotter, Johannes Gutenberg University Mainz. After delivery in the animal facility those animals were kept under the same circumstances as mentioned above.

3.8. Applied computer software

Program	Company
Adobe Photoshop CS2	Adobe Systems, München (Germany)
GraphPad Prism 4.0	GraphPad Software, San Diego (USA)
IPA ® Ingenuity Pathway analyses software	Ingenuity systems, Redwood City (USA)
Leica image capture software	Leica Microsystems, Wetzlar (Germany)
Microsoft Office	Microsoft, (USA)
Partek Genomics Suite software	Partek Inc., St. Louis (USA)
SMART 2.5.5	Panlab, Barcelona, Spain
SPSS 19.0 v statistics	SPSS Inc., Herrenberg (Germany)

3.9. Gene array

GeneChip Mouse Genome 430A 2.0 Array from Affymetrix was performed at the Institute for Translational Oncology (TRON), Mainz (Germany).

4. Methods

4.1 Molecular biology

4.1.1 Genotyping

To determine the genotype of the mice, PCR reactions were run on genomic DNA samples prepared from tail biopsies. The presence of the Cre-recombinase allele marked the mutant (conditional knock-out) mice and the absence of this allele the wild-type mice. All CB1 conditional mouse lines were genotyped in our laboratory, while the genotyping of the NG2-EYFP animals was done in the laboratory of Professor J. Trotter.

After cutting off a small piece of tail of the mice, biopsies were incubated overnight at 56°C in 500 µl lysis buffer containing 0.5 mg/ml Proteinase K in a thermomixer shaking at 850 rpm. The next morning, samples were centrifuged at room temperature for 10 min at 13.000 rpm. Immediately after centrifugation, about 500 µl of the top phase of the probes were transferred into new tubes filled with 500 µl isopropanol and mixed carefully by inversion. At this time the DNA precipitates. Afterwards probes were centrifuged again at room temperature for 10 min at 13.000 rpm. The supernatant was removed with a pipette, 300 µl of 70% ethanol was added and the tube was mixed by inversion. Again the supernatant was removed and the pellets were left to air dry for approximately one hour. Lastly, the pellets were dissolved in 200 µl TE buffer by vortexing carefully and again shaken for one hour at 60°C and two hours at 37°C. The dissolved DNA was kept at 4°C until further use.

For the PCR, a master mix was prepared which included for each reaction 5.0 µl Green GoTaq® Flexi buffer, 2.5 µl Mg_2Cl, 1 µl dNTPs, 1.25 µl of primer G 100 and G 101, 1.75 µl of primer G 50 and G 51, 0.125 µl GoTaq polymerase and 9.375 µl autoclaved H_2O. At last 1 µl DNA was added and all probes were put into the Biometra T3000 thermocycler. The following PCR program was used:

$CB1^{f/f;D1\text{-}Cre}$, J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$,$CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ mouse line

» 95°C for 3 min
» 30x [95°C for 1 min; 55°C for 1 min; 72°C for 1 min]
» 72°C for 3 min

10 µl of PCR reaction was applied on a 1.5% TBE agarose gel for gel electrophoresis. The following bands show the possible results:

Cre +	about 350 bp	**f/f**	500 bp
Cre -	no band	**f/+**	500 bp and about 400 bp
		+/+	about 400 bp

CB1 null-mutant mouse lines (J-CB1$^{-/-}$ and CB1$^{-/-}$)

» 95°C for 5 min
» 30x [95°C for 45 sec; 55°C for 45 sec; 72°C for 45 sec]
» 72°C for 3 min

10 µl of PCR reaction was applied on a 1.5% agarose/TBE gel for gel electrophoresis started. The following bands show the possible results:

CB1 $^{-/+}$ (M/WT)	413 bp and 342 bp
CB1 $^{+/+}$ (WT/WT)	413 bp
CB1 $^{-/-}$ (M/M)	342 bp

4.2. Gene array

GeneChip Mouse Genome 430A 2.0 Array from Affymetrix was performed at the Institute for Translational Oncology (TRON), Mainz (Germany).
The GeneChip® Mouse Genome 430A 2.0 Array is a single array that contains over 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes that can be used to explore mechanisms behind biological and disease processes. Sequences used in the design of the array were selected from GenBank®, dbEST, and RefSeq. The sequence clusters were created from the UniGene database (Build 107, June 2002) and then refined by analysis and comparison with the publicly available draft assembly of the mouse genome from the Whitehead Institute Center for Genome Research (MSCG, April 2002). Oligonucleotide probes complementary to each corresponding sequence are synthesized in situ on the array. Eleven pairs of oligonucleotide probes are used to measure the level of transcription of each sequence represented on the GeneChip Mouse Genome 430A 2.0 Array (Mouse 430A 2.0) (http://media.affymetrix.com/support/technical/datasheets/mogarrays_datasheet.pdf).

4.2.1 Preparation of probes

In order to gain further insight in processes in the brain of CB1$^{f/f;D1\text{-}Cre}$ mice after a conditioned place preference (CPP) experiment with nicotine injections, the animals were immediately sacrificed after data collection in the post test.
Data analysis revealed the specific behaviour of the mice. Based on that behaviour CB1$^{f/f;D1\text{-}Cre}$ mice and their wild-type littermates were grouped as follows:

CB1$^{f/f;D1\text{-}Cre}$ WT	**CB1$^{f/f;D1\text{-}Cre}$ KO**
Naïve, untreated	Naïve, untreated
Saline treated (controls)	Saline treated (controls)
Nicotine treated, showing preference to drug	Nicotine treated, showing preference to drug
Nicotine treated, showing aversion to drug	Nicotine treated, showing aversion to drug

Table 1: Arrangement of groups based on behaviour of CB1$^{f/f;D1\text{-}Cre}$ mice shown in conditioned place preference experiment (n=3/group, 12 animals per genotype)

In order to compare candidate genes from this experiment with the CB1$^{f/f;D1\text{-}Cre}$ mouse line we performed another CPP experiment with nicotine injections with the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line following the same protocol.

4.2.1.1 Brain isolation

Animals were killed by over-anesthetizing them with isofluran. Afterwards mice were decapitated and the fur and most of the flesh were parted from the skull. First the skull was opened with two horizontal sections from the foramen magnum and one median cut. Bone parts were carefully removed until the brain was exposed. The brain was taken out and frozen onto the cerebellum on aluminium foil on dry ice. The isolated brains were stored at -80°C until further use.

4.2.1.2 Punches

First, the brain was mounted onto the cerebellum and cut until the beginning of caudate putamen (bregma 1.54 mm, interaural 5.34 mm) with the help of the atlas of Paxinos and Franklin (1997). Brain slices were stained with toluidine blue to verify the position. With a puncher, two punches of each brain hemisphere in the area of caudate putamen were taken ending at bregma -0.23 mm, interaural 3.58 mm. Each punch had a volume of 1-2 mm^3. Additionally for the experiments with the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals, punches from the striatum were processed. For documentation every fifth brain slice was taken and stained until the holes in the slices could not be seen anymore.

4.2.1.3 Purification of total RNA

For preparation of the total RNA punches of the right hemisphere were used and homogenized with Tissue LyserII from Qiagen for 30 sec at a frequency of 1/30. The protocol “Purification of total RNA from animal tissue” from QIAGEN RNeasy Mini kit was exactly followed skipping the DNase I digest between step 6 and 8. OD ratio was measured with Nanodrop from peqlab.

Amplification of probes with Ambion MessageAmp™ Premier RNA Amplification Kit from Applied Biosystems was performed in the Institute for Translational Oncology (TRON), Mainz (Germany).

4.2.2 Validation of candidate genes

Analysis of raw Affymetrix gene array data was performed at the Institute for Translational Oncology (TRON). Bioinformatics processed the microarray data of 22 Affymetrix microarrays (GeneChip Mouse Genome 430A 2.0) by the RMA algorithm (Irizarry et al., 2003) implemented in the Bioconductor R package (Gentleman et al., 2004; R Development Core Team, 2010). For this, custom probe annotation was added (Dai et al., 2005). For comparison of the expression data, the R package limma (Smyth, 2004) was used to estimate fold changes for the defined contrasts and calculate false discovery rates (FDRs) by the Benjamini and Hochberg method (1995).

Additionally, the raw data of the gene array was analysed with special Partek Genomics Suite program at the TRON institute. Pathway analyses were made with the IPA ® Ingenuity Pathway analyses software (www.ingenuity.com). These analyses were based only on p-value, not FDR value. Therefore, we selected only those genes that we could have found also with the more stringent data sets analysed by the bioinformatics institute from TRON.

4.2.2.1 Selection of candidate genes

With the GeneChip® Mouse Genome 430A 2.0 Array 22,600 probe sets, representing transcripts and variants from over 14,000 well-characterized mouse genes, were tested. First selection criterion was the distribution in the parameters genotype, treatment and behaviour. This lead to the groups found in Tab. 2. Each group contained three animals.

Genotype	$CB1^{f/f;D1\text{-}Cre}$ WT	$CB1^{f/f;D1\text{-}Cre}$ KO	$CB1^{f/f;D1\text{-}Cre}$ WT	$CB1^{f/f;D1\text{-}Cre}$ KO	$CB1^{f/f;D1\text{-}Cre}$ WT	$CB1^{f/f;D1\text{-}Cre}$ KO
Treatment	Nicotine	Nicotine	Saline	Saline	Untreated	Untreated
Behaviour in CPP	Preference	Preference				
	Aversion	Aversion				

Table 2: Arrangement of groups based on their treatment and the behaviour shown in conditioned place preference experiment (n=3/group, 12 animals per genotype)

For the basal phenotype of the animals, I compared $CB1^{f/f;D1\text{-}Cre}$ WT untreated with $CB1^{f/f;D1\text{-}Cre}$ KO untreated. The stress induced phenotype was evaluated by the comparison of $CB1^{f/f;D1\text{-}Cre}$ WT saline injected versus $CB1^{f/f;D1\text{-}Cre}$ WT untreated and by $CB1^{f/f;D1\text{-}Cre}$ KO saline injected versus $CB1^{f/f;D1\text{-}Cre}$ KO untreated. Nicotine induced changes of phenotype were either tested for genotypes with the same behaviour ($CB1^{f/f;D1\text{-}Cre}$ WT nicotine injected with preference compared to $CB1^{f/f;D1\text{-}Cre}$ KO nicotine injected with preference; $CB1^{f/f;D1\text{-}Cre}$ WT nicotine injected with aversion compared to $CB1^{f/f;D1\text{-}Cre}$ KO nicotine injected with aversion and both saline treated genotypes) or in the genotype group with different behaviours for of same treatment ($CB1^{f/f;D1\text{-}Cre}$ WT nicotine injected with preference compared to $CB1^{f/f;D1\text{-}Cre}$ WT nicotine injected with aversion; $CB1^{f/f;D1\text{-}Cre}$ KO nicotine injected with preference compared to $CB1^{f/f;D1\text{-}Cre}$ KO nicotine injected with aversion). Additionally saline treated genotypes were compared to each other ($CB1^{f/f;D1\text{-}Cre}$ WT saline injected compared to $CB1^{f/f;D1\text{-}Cre}$ KO saline injected) and to the corresponding behaviour in the genotype ($CB1^{f/f;D1\text{-}Cre}$ WT saline injected compared to $CB1^{f/f;D1\text{-}Cre}$ WT nicotine injected with preference and to $CB1^{f/f;D1\text{-}Cre}$ WT nicotine injected with aversion). With this scheme, 13 comparisons were performed.

Group 1	WT untreated – KO untreated
Group 2	WT untreated – WT saline treated
Group 3	KO untreated – KO saline treated
Group 4	WT saline treated- KO saline treated
Group 5	WT nicotine treated preference - KO nicotine treated preference
Group 6	WT nicotine treated aversion - KO nicotine treated aversion
Group 7	WT saline treated - WT nicotine treated preference
Group 8	WT saline treated -WT nicotine treated aversion
Group 9	WT nicotine treated preference- WT nicotine treated aversion
Group 10	KO saline treated - KO nicotine treated preference
Group 11	KO saline treated - KO nicotine treated aversion
Group 12	KO nicotine treated preference- KO nicotine treated aversion
Group 13	WT nicotine treated preference- KO nicotine treated aversion

Table 3: Arrangement of groups received from comparison of different genotypes based on their treatment and the behaviour shown in conditioned place preference experiment (n=3/group, 12 animals per genotype)

For each group 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes were tested. We filtered the 15 strongest up- or downregulated genes and compared the results with the genes from the analysis with the Partek Genomics Suite program. Out of this comparison we finally decided for a maximum of 9 genes of interest, one housekeeping gene (glucoronidase beta, *gusb*) and *cnr1* as positive control.

4.2.2.2 Gene validation by quantitative real-time polymerase chain reaction

The genes, chosen from the gene array were validated by quantitative real-time polymerase chain reaction (qPCR) with the T7300 system from Applied Biosystems. The ability to magnify trace nucleic acids by using PCR amplification offered many possibilities in the field of science. The development of real-time PCR brought the applications for PCR technology forward. With the help of the enzyme reverse transcriptase it is now possible to convert mRNA transcripts from the whole genome to cDNA. Together with real-time PCR analysis, it is now possible to measure the quantity of DNA copies being amplified exactly.

4.2.2.2.1 Purification of total RNA

For validation of candidate genes by qPCR, we purified the total RNA of both punches of the left hemisphere of the 18 animals after CPP and the 6 untreated animals. Afterwards we homogenized the material with Tissue LyserII from Qiagen for 30 sec at a frequency of 1/30. The protocol “Purification of total RNA from animal tissue” from QIAGEN RNeasy Mini kit was exactly followed, this time including the DNase I digest between step 6 and 8. OD ratio was measured with Nanodrop (Peqlab).

With the punches from the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals after CPP and 9 untreated animals of that line we dealt in the same way.

4.2.2.2.2 Converting RNA into cDNA

The total RNA of all probes was converted directly after purification into cDNA with the help of “High capacity cDNA reverse transcription Kit” from Applied Biosystems. We followed the protocol accurately. Probes were stored at -20°C until use.

4.2.2.2.3 Preparation of plates

A 96-well plate was used for each gene tested. Duplicates from all 24 probes and two non-template controls (NTC) were used for the experiments with CB1$^{f/f;D1\text{-}Cre}$ animals. For the preparation of MasterMix (MM) for all probes we calculated with an overpipette of 11 %. Therefore, we prepared 56 fold MM for each plate. MM per well always contained 10 µl of 2x TaqMan Gene expression Master Mix and 1 µl of TaqMan primer/probe Mix. Additionally, we pipetted 9 µl of cDNA template into each well. This led to the following pipetting scheme. The numbers in the scheme represent the animal identification.

A	1	1	2	2	3	3	4	4	5	5	6	6
B												
C	7	7	8	8	9	9	10	10	11	11	12	12
D												
E	13	13	14	14	15	15	16	16	17	17	18	18
F												
G	19	19	20	20	21	21	22	22	23	23	24	24
H	NTC	NTC										

Scheme 1: Pipetting scheme for qPCR. For validation of genes found with the GeneChip® Mouse Genome 430A 2.0 Array in CB1$^{f/f;D1\text{-}Cre}$ animals

Separately, for the experiments with J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals, we used duplicates from 19 probes (19 mice, punches from striatum) and two non-template controls (NTC). For the preparation of MasterMix (MM) for all probes we calculated with an overpipette of 11%. Therefore we prepared 95 fold MM for each plate. MM per well always contained 10 µl of 2x TaqMan Gene expression Master Mix and 1 µl of TaqMan primer/probe Mix. Additionally, we pipetted 9 µl of cDNA template into each well. This led to the subsequent pipetting scheme. The numbers in the scheme represent the animal identification.

A	1	1	2	2	3	3	4	4	5	5	6	6
B												
C	7	7	8	8	9	9	10	10	11	11	12	12
D												
E	13	13	14	14	15	15	16	16	17	17	18	18
F												
G	19	19										
H	NTC	NTC										

Scheme 2: Pipetting scheme for qPCR. For additional validation of the genes found with the GeneChip® Mouse Genome 430A 2.0 Array in CB1$^{f/f;D1\text{-}Cre}$ animals, now validated in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice

Further qPCR plates followed the same principle in pipetting as presented in scheme 1 and scheme 2.

4.2.2.2.4 qPCR program

The program was set with the manual delivered with the qPCR machine T7300 from Applied Biosystems. TaqMan® Gene Expression Assays consist of a fluorescein FAM™ dye-labelled TaqMan® MGB probe and two unlabelled PCR primers. For the FAM™ dye, we used the following program:

Stage 1: 50°C, 2.00 min (Uracil_N-Glycosylase (UNG) activation)

Stage 2: 95°C, 15.00 min (Taq Polymerase activation, UNG deactivation

Stage 3: 95°C, 15.00 sec (denaturation)
60°C, 1.00 min (annealing /extension; temperature depending on primer)

The PCR program runs over 40 cycles. Data was collected at stage 3 in step 2, processed with Microsoft Excel, using the housekeeping gene *gusb* as the reference gene, and analysed with SPSS 19.0 v statistics, based on the $2^{-\Delta\Delta CT}$ method. The $2^{-\Delta\Delta CT}$ method is a convenient way to analyse the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen, 2001).

4.3 Behavioural analyses

Behavioural experiments are performed to find out whether animals with genetic alterations show behavioural differences as compared to their wild-type littermates. During the last two decades, a lot of different assays for rodents have been established to assess numerous behavioural domains, such as motor functions, sensory abilities, social behaviour or emotional and spatial learning. It is known that genes and environment are both important and interdependent determinants of behavioural responses (de Sousa et al. 2006). Therefore, behavioural neuroscience and molecular genetics go hand in hand, and both fields of research focus on the roles of genes on behaviour.

4.3.1 Characterisation of basic functions

The first step in behavioural phenotyping is to test whether the animals have general abnormalities or deficits which will obviously limit our planned experiments. A mouse with deficits in locomotion, e.g. because of weight differences between genotypes, will not be able to perform in a lot of behavioural challenges. Neither will a deaf animal react to tones in auditory tasks. Therefore, it is obligatory that the phenotype of a mouse line should first be checked in its general home cage behaviour.

4.3.1.1 Measurement of body weight

Animals that were chosen for an experiment were weighed before start of tests in order to avoid locomotion alterations by weight differences. Each mouse was placed in a beaker and weighed with a standard laboratory balance. Data was collected and analysed with GraphPad Prism 4.0. Results are shown for $CB1^{f/f;D1\text{-}Cre}$ mouse line of our laboratory and for the NG2-EYFP mouse line from the laboratory of Professor J. Trotter.

4.3.1.2 Acoustic startle response

The acoustic startle response test provides a good measure of gross hearing ability (Crawley, 1999). Acoustic startle is a reflex that can be seen in most mammals. It is a yes-no-response measured by a human observer. By presenting a loud tone, the observer checks the reaction of the animals based on their body flinch and vocalization.

This behavioural test was used to characterize the NG2-EYFP mouse line. The mouse was put inside the cylinder of fear conditioning boxes (Fig. 11) and was habituated for 5 min with lights switched on inside the boxes. Then, a tone of 95 dB was presented for 2 seconds and the reaction of the animal was observed.

Fig. 11: Fear conditioning cylinder to measure acoustic startle

4.3.2 Models of locomotion and motor functions

4.3.2.1 Open field test

The most standardized measurement of locomotion and motor functions is the activity of mice in the open field within a predetermined time period. In the open field test, the baseline levels of locomotor activity in transgenic or knock-out mice can be compared to their wild-

type littermate controls. This model can also be used to show differences in behaviour influenced by administered drugs.

In this study, mice of the conditional mutant lines $CB1^{f/f;D1\text{-}Cre}$, $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ and animals of the NG2-EYFP mouse line were placed in the centre of a white plastic chamber measuring 40 x 40 x 40 cm, which was built in the institute's workshop (Fig. 12). Four boxes were used at the same time. Per box, one mouse was left for 30 minutes to explore the novel environment and was monitored by an automated video tracking computer program (Smart, PanLab; Barcelona, Spain). The Smart program worked with the following parameters. For detection, the static position was used. Saturation, threshold, hue and erosion were adjusted to 40, 50, 6 and 0 respectively. The experimental area was lit with a special lighting system, which made it possible to arrange the experiment under optimized conditions. The light on the floor of the boxes had the intensity of around 100 lux. After the test, the animal was removed from the chamber and returned to its home cage.

Fig. 12: Open field box, used for testing locomotion

4.3.2.2 Grip strength test

Measuring the grip strength is a task, which assesses neuromuscular function and muscular strength by sensing the peak amount of force that is required to make a mouse release its grip. Forelimb grip strength is measured as tension force using a grip strength meter (GSM).

For this paradigm, a Grip Strength Meter (GSM) from TSE systems was used, which consists of a baseplate, a stainless steel grip/metal bar and a force sensor, which measured the data of NG2-EYFP mice. The data was written down and analysed on a different computer. No special light conditions were used for the procedure, only standard room light. By lifting the mouse over the baseplate by the tail, the animal was able to grasp onto the steel grip with its

forepaws. The tail of the mouse was then adjusted to the centre of the grid and the mouse was gently pulled backward by the tail until it released the grip to the bar. The GSM measured the maximal force before the mouse loses the contact to the bar. Three trials were performed for each mouse with a 1-minute resting period between trials. For analyses the mean of all three measures was used.

4.3.2.3 Rotarod test

Motor coordination and balance and motor learning of mice can be measured by an assay on the rotarod. As it is shown in Fig. 12, the rotarod apparatus is a rotating cylinder of approximately three centimetres in diameter, where five mice can be placed at the same time. Motor learning is evaluated by repeated daily testing on the rotarod. Reduction in latency to fall down, normally over repeated testing sessions, provides a measure of cerebellar learning (Crawley, 1999)

In this study, a rotarod apparatus from Ugo Basile (Italy) was used (Fig. 13), as previously described (Bilkei-Gorzo et al., 2005; Serradj et al., 2006). This apparatus is 38 cm high, 44 cm long and 32 cm deep. It consists of five 3 cm diametric rotors. Six flanges divide the rotor into five lanes, so that five mice can walk simultaneously on the treadmill without seeing each other.

Fig. 13: Rotarod apparatus

NG2-EYFP mice and their respective wild-type littermates with the age of 8 – 12 months were tested. Genotype groups contained 18 wild-type and 20 knock-out male mice. The animals were placed onto the cylinder at the speed of 4 rpm.

Afterwards, the speed of the rod accelerated from 4 to 40 rpm over 300 sec. The maximum speed was reached after 289 sec and remained constant until the end of the trial. Time until the animals fell down was registered by the apparatus. The cut-off time for each trial was 300 seconds. The animals were allowed to rest for five min between two trials and were tested for three trials per session, one session per day. The experiment lasted three days in all. The experimenter was blind to the genotype during the experiment and the animals were equally distributed, so that both genotypes were always tested in parallel. The $CB1^{f/f;D1\text{-}Cre}$ mice, aged 4 – 5.5 months, were tested with the same apparatus and the same paradigm, except the maximum speed was in this experiment 80 rpm.

4.3.3 Models of emotion

4.3.3.1 Elevated plus maze test

The elevated plus maze (EPM) is based on a naturalistic conflict of the animals between the tendency to explore a novel environment and the aversion to a brightly lit and open area. Further, in this model new components are added such as height and openness. The run-ways or “arms” of the EPM exhibit a specific height and openness (Carobrez and Bertoglio, 2005).

The EPM used was built in the institute´s workshop and made of white and black plastic. Its height was 100 cm and it had two closed and two open arms of 35 cm length and 6 cm width (Fig. 14). The enclosed arms had additional black plastic walls of 20 cm height. For the experiment, a mouse was placed in the centre between open and enclosed arms and was left there to explore all areas for 15 minutes. A camera and video tracking system recorded the path of the mouse, and the time spent in open and enclosed arms and the total amount of entries in both were measured.

The Smart computer program worked with the following parameters. For detection, the black position was used. Saturation, threshold, hue and erosion were adjusted to 40, 50, 6 and 2 respectively. The experimental area was lit with a special lighting system, which made it possible to arrange the experiment under optimized conditions. The light on the floor of the open arms and the centre had an intensity of around 200 lux, while the light on the enclosed arms had about 140 lux. Every session was recorded on DVD.

4.3.3.2 Light-dark test

Similar to the EPM, the light-dark test (LD) is based on the natural conflict between the tendency to explore unknown areas and the aversion of a brightly lit open field.

The light-dark-boxes used were made of white and black plastic and built in the in-house workshop. In Fig. 15, an example of one box is shown. For an experiment, four boxes were used. All boxes had the dimensions of 26 x 38 cm for the lit compartments and 13 x 38 cm for the dark compartments and were 30 cm high. The white compartments were open, while the dark ones were closed. At the beginning of the experiment, one mouse was placed into the centre of the lit compartment and the recording with the camera and video tracking system started. When the animal entered the dark box with all four paws, the five minutes experimental time started. While the time was running, every four-paw entry of the mouse into the white area and the whole time it stayed there was recorded. The detection with the Smart computer program worked under the following conditions. For detection, the black position was used. Saturation, threshold, hue and erosion were adjusted to 40, 150, 6 and 0 respectively. A special lighting system was used to light the experimental area. With the help of this light system it was possible to arrange the experiment under optimized conditions. The light intensity on the floor of the lit compartment was around 100 lux. Every session was recorded on DVD in order to manually analyse the experiment later.

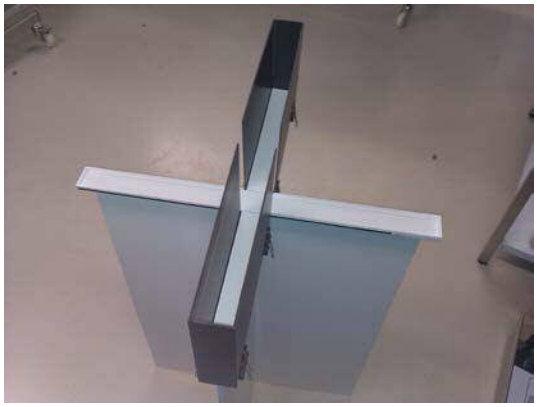

Fig. 14: Elevated plus maze

Fig. 15: Light-dark box

4.3.3.3 Forced swim test

The FST is the most commonly used test for assessment of depression-like behaviour in animal models. The test is commonly used to measure the effect of antidepressant drugs on behaviour, but can reveal a basal difference in depression-like behaviour between genotypes as well. It is based on the fact that mice swim in the water to seek an escape route (Crawley,

1999). If the animal realizes there is no escape from the water, it stops swimming and will start floating on the water surface.

The forced swim test was carried out using a procedure modified from the original report by Porsolt et al (1977). The mice of NG2-EYFP mouse line were individually placed into a glass beaker (volume 2000 ml, 25 cm height, 10 cm diameter) containing approximately 1000 ml of water maintained at 24–26°C. The experiment was recorded for a total time of 6 min. Then, the last 4 minutes of the video file were analysed and each animal was rated for immobility time, defined as the absence of active, escape-oriented behaviours such as swimming, jumping, rearing and the time spent floating.

4.3.4 Models of recognition memory and learning

4.3.4.1 Novel object recognition task

The novel object recognition task (NORT) is used to evaluate recognition memory in rodent models. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory. NORT combines a general exploration test with a visual recognition memory paradigm. Therefore, it is used to evaluate object exploration and object recognition. The test was performed in a white plastic open field chamber (H40 cm x W40 cm x L40 cm). The protocol used was modified from Ennaceur and Delacour (1988), Tang et al. (1999) and Tordera et al. (2007). With this paradigm we studied the behaviour of our conditional mutant lines $CB1^{f/f;D1\text{-}Cre}$, $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ and of animals from the NG2-EYFP mouse line.

For habituation, a mouse was placed into the empty open field and allowed to explore the box for 10 min once a day for two days. The first habituation session was analysed according to a standard open field paradigm (Fig. 16), hence, total distance moved and time spent in the centre was evaluated using SMART software (PanLab, Spain). On day 3, two identical objects (A left, and A right; two metal cubes with H4 cm x W3 cm x L5 cm) were placed symmetrically 6-7 cm from the walls and separated 16-18 cm from each other. The mouse was placed into the box at an equal distance from both objects and DVD-recorded for 10 min. After this first exposure to the object, the mouse was returned to its home cage. Two h and 24 h later, the mouse was placed into the open field again and exposed to the familiar object (A) and to a novel object (B for the 2 h time point (Fig. 16), and C for the 24 h time point, respectively) each time for 10 min (retention tests). The novel object B was a plastic billiard ball (5.72 cm diameter) fixed on a metal plate (0.2 cm) and C was a round glass flask

(H6 cm x W3 cm), filled with sand and closed with a black rubber plug. The familiar object A was always positioned on the left side, while the new object was on the right side. Box and objects were cleaned with 70% ethanol after each trial to avoid olfactory cues. The experiment was DVD-recorded and the total time that the animal spent exploring each of the two objects in the training and retention phases was evaluated by an experimenter blind to the genotype. Object exploration was defined as the orientation of the nose pointing directly to the object at a distance <2 cm and/or touching the object with the nose and whiskers. Time spent climbing and sitting on the object was not regarded as exploration, and was therefore excluded from measurement (Ennaceur and Delacour, 1988), as these activities do not represent a form of exploration. The discrimination index (DI) was calculated as the difference between the time spent exploring the new (N) and familiar (F) object, divided by the total time exploring the objects [(N - F) / (N + F)]. A positive DI is considered to reflect increased memory retention of the familiar object (Tordera et al. 2007).

Fig. 16: Pictures of an open field with two equal objects for the NORT on left panel and a mouse exploring the familiar and a new object on the right panel

4.3.4.2 Morris water maze task

The Morris water maze task (MWM) is one of the most popular tasks in behavioural neuroscience. In its most basic form, the water maze assesses spatial learning and memory. Performance in the MWM is acutely sensitive to changes in the hippocampus function. The task is based on the principle that mice are highly motivated to escape from a water environment by the quickest and direct route (Crawley, 1999).

We used a protocol modified from the original protocol by Richard Morris (1984). A circular white plastic pool with 1.50 m diameter and 0.60 m height was filled with approximately 0.8m³ of water and made opaque with 2-4l of milk (Fig. 17). A 30 - 40 cm high, 10 cm diameter Plexiglas platform was used. Four visual black cues on white room walls divided the pool

into four equal quadrants (N, S, W, and E) to give the animals the possibility to orientate. The water maze was exactly adjusted under the camera/detection system and (re)calibrated with the Smart program. The pool was filled one day before the experiment, and the water was kept between 20-23 °C. Milk was added directly before the experiment. After finishing the experiment, the pool was cleaned immediately to avoid distraction of animals by odour, because the milk started to stink soon.

On the first day of the experiment, animals of the NG2-EYFP mouse line were familiarized with the water maze and a hidden platform for one trial. At this trial, the cues were not put on the walls yet. The mouse was put inside the pool for the whole possible distance (diameter) and had to swim 2 minutes. If it was not able to find the hidden platform, it was lead to it and stayed there for another minute.

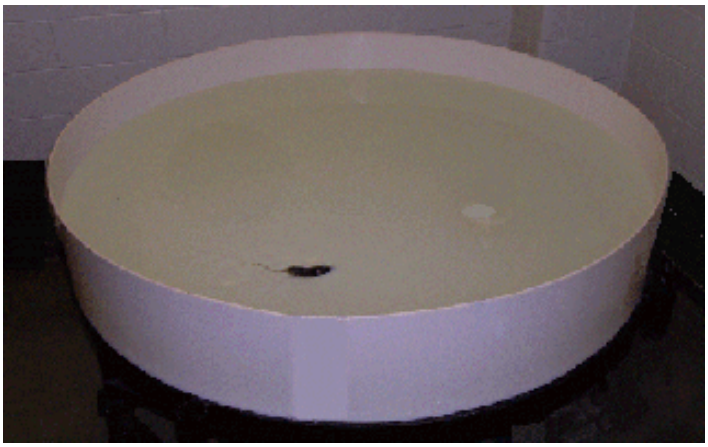

Fig. 17: Morris water maze pool taken from www.watermaze.org

Before starting the training sessions, the visual cues were placed onto the four walls surrounding the maze. The training sessions started directly after the familiarization at day one and went until day five. Four trials per day were performed. At each trial, a time period of 60 sec was measured. If the mouse reached the platform within this time period, the time was automatically stopped after 5 sec on the target. If it was not able to reach the platform, it was put on it after the end of the trial and stayed there for 10 sec. For all trials, the platform stayed in the same quadrant, but the animals were randomly released from four different positions (N, S, E, W; position changed every day) of the pool perimeter.

Day six started with the probe trial. The platform was removed and the mouse was put into the water at a new position (NE). Crossings at the position of the removed platform and the time spent in the quadrant zone of the platform during the whole 60 sec were recorded. If there were no crossings over the target, time was stopped also after 60 sec. The Smart pro-

gram was used to measure the escape latency, path length and the time spent in each quadrant of the animal during each trial.

4.3.4.3 Sociability test

For this paradigm a modified sociability test was used, which was based on the protocol of Moy et al. (2004) in order to detect strain differences between the $CB1^{f/f;D1\text{-}Cre}$, $CB1^{f/f;dlx5/6\text{-}Cre}$ and $CB1^{f/f;NEX\text{-}Cre}$ mouse lines and to reveal genotype differences in each line.

The applied test chamber (H41 cm x W42 cm x L70 cm) was divided into three compartments (H40 cm x W40 cm x L22 cm), all accessible by doors (H7.5 cm x W10 cm) in the dividing walls (Fig. 18). Chambers and cages were cleaned with 70% ethanol after each trial to avoid olfactory cues. The whole experiment was DVD-recorded, and the total time that the test animals spent in each of the compartments during the sociability and the social novelty phase was measured by SMART software (PanLab, Spain). Male C57BL/6N animals (10-12 weeks old) were used as interaction partners for the sociability and social novelty phase.

Habituation phase: The mouse was placed into the middle compartment for 5 min with the entries to the side compartments blocked and was allowed to explore the box.

Sociability phase: After the habituation phase, the entries were opened and the mouse had free access to both side compartments for 10 minutes. By doing this, the animal tested was exposed to a novel C57BL/6N interaction partner and a novel object (round cage described below), positioned in the two side compartments. The position of the interaction partner (left vs. right compartment) was alternated between trials to avoid any bias. The interaction partner itself was enclosed in a round cage (10 cm in diameter; 30 cm high, where the upper part consisted of 20 cm Plexiglas and the 10 cm of the lower part was covered by metal bars, one cm apart, to allow interaction but to prevent fighting; Fig. 17). To minimize stress levels of the animals used as interaction partners, they were habituated to the cages four times for 10 min on two days prior to the actual test days. To counterbalance individual differences of these interaction partners they were equally used for wild-type and mutant test mice. The novel object control (empty cage) was always positioned in the opposite compartment to the cage with the interaction partner. The discrimination index (DI) was calculated as difference between the time spent exploring the novel object (nO) and novel animal (nA), divided by the total time exploring both [(nO - nA)／(nO + nA)]. A positive DI is considered to reflect a preference for the social interaction partner.

Social novelty phase: 2 h after the sociability phase, another, unknown interaction partner (novel) was put inside the box into the former empty cage. The interaction partner from the sociability phase (familiar) was again placed into the same cage and same compartment as

before. The cage with the novel animal was positioned in the other side compartment. Doors were opened. The test animal was placed into the middle compartment and the free exploration period of 10 min started. The DI was calculated as in the sociability phase based on the difference between the time spent exploring the new (N) and the familiar (F) animal, divided by the total time exploring both [(N - F) / (N + F)]. A positive DI is considered to reflect memory retention for the familiar animal.

Fig. 18: sociability box with three compartments and the compartment with the interaction partner

4.3.4.4 Resident-intruder test

The resident-intruder test was performed by placing a novel, group housed intruder mouse (C57BL/6N) into the home cage of the single-housed animal ($CB1^{f/f;D1\text{-}Cre}$, $CB1^{f/f;dlx5/6\text{-}Cre}$ or $CB1^{f/f;NEX\text{-}Cre}$ mouse) for 10 min. This paradigm allows evaluating social exploration and aggressive behaviour (Goyens & Noirot, 1975). To decrease interactions induced by the intruder, younger animals (males, 11-13 weeks) were used as intruders. The experiment was DVD-recorded, and the total interaction time of the animals spent exploring was measured. Interaction was defined by any type of physical interaction clearly directed towards the partner. Duration, percentage of time and number of fights were evaluated separately. Fighting was defined by physical struggling between the interaction partners initiated by an attack of the resident towards the intruder.

4.3.5 Models of addiction

4.3.5.1 Conditioned place preference test

The most commonly used apparatuses to carry out the conditioned place preference (CPP) test have been either two- or three-compartment 'conditioning boxes'. In these boxes, one compartment is associated with drug injections, whereas the other is associated with vehicle administration. The experiment normally starts with a pre-test where the animal is allowed to explore all compartments without any drug treatment. This is necessary to find out if there is a natural preference for one of the compartments. Afterwards, repeated pairings of a changing drug/vehicle administration protocol will follow, and the experiment ends with a test day, the so called post-test, in a drug-free state, at which the animal will be allowed to move freely across all compartments again under the same conditions as on the pre-test day. The increase in time spent in the drug-associated compartment is considered as a measure of conditioned place preference.

Different surface consistency, variable colours of boxes and different kinds of (grid) floors lead to the so-called *unbiased* and *biased* CPP procedures. In *unbiased* CPP procedures, there is no drastic difference between the compartments and no natural preference in animals is caused. *Biased* CPP procedures are different in most of the characteristics mentioned above. For this reason, one can see a natural preference in animals in this model. In *unbiased* CPP, injection is associated with one arbitrarily chosen compartment, whereas regarding the *biased* CPP, the drug is paired with the non-preferred compartment, and CPP is measured as overcoming the initial aversion of that environment.

Fig. 19: CPP apparatus during the test

As shown in Fig. 19, a biased CPP was used in this study. The protocol was described earlier (Castane et al., 2002; Maldonado et al. 1997). Four CPP boxes were used at the same time. One CPP apparatus consisted of one white, one grey and one black plastic box which were linked with doors. The dimensions of the black and white boxes were 22 x 22 x 25 cm, of the grey boxes 11 x 22 x 25 cm. All boxes were 22 cm high from the grid floor. Inside the black box a grid floor consisting of 2 mm stainless-steel rods 1 cm apart (from each other) was present, while inside the white box the grid floor consisted of a net of 1 mm stainless-steel rods, which were arranged in 7 x 7 mm grids. The ground under all grid floors was covered with sawdust. During the conditioning phase, the mouse received, depending on experiment, either nicotine (0.5 mg/kg) or cocaine hydrochloride (10.0 mg/kg i.p.) in the naturally non-preferred box and vehicle i.p. in the preferred box. By use of this paradigm, differences in addiction-like behaviour of the $CB1^{f/f;D1\text{-}Cre}$ and J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mouse lines were evaluated. Additionally, we tested the null-mutant CB1 mouse lines ($CB1^{-/-}$ and J-$CB1^{-/-}$). Pre-test experiments were always done with the respective wild-type C57BL/6N or C57BL/6J background strains.

The mouse received the first injection in the morning, while the second injection followed at least 4 hours later on the same day. Compartment and treatment (drug vs. vehicle) changed every day over the conditioning phase. After injection, the animal stayed in its dedicated compartment for 20 min. The control animals received vehicle every day independent of the compartment.

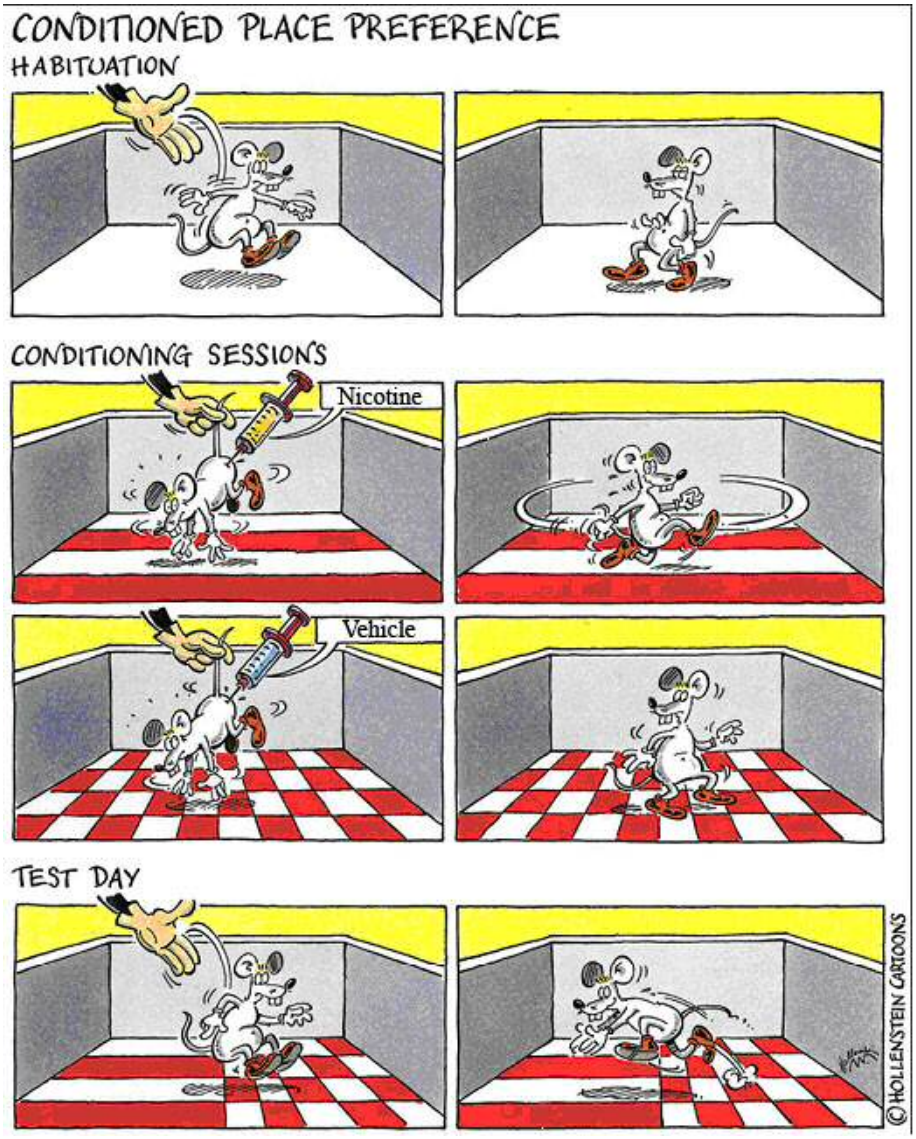

Fig. 20: Conditioning place preference in a biased system –
Modified from Sanchis-Segura and Spanagel, 2006

4.3.5.2 Conditioned place aversion test

The conditioned place aversion (CPA) test was used to measure aversive effects of drugs or withdrawal after chronic drug treatment. In the latter case, it was important that the animal was put into a chronically treated ("addicted") state. CPA data should substantiate results of the CPP. In the CPA test, same boxes are used as for the CPP test. Here as well, one compartment was associated with drug injections, whereas the other was used after vehicle administration. The experiment started with a pre-test where the animal was allowed to explore all compartments without any injections. This is necessary to find out if there is a natural preference for one compartment. Afterwards, one day of a changing drug/vehicle administration protocol will follow, and the experiment closes with an injection-free post-test on the third

day, at which the animal will be allowed to move freely across all compartments again under the same conditions as on the pre-test day. The decrease in the time spent in the drug-associated compartment was considered as a measure of conditioned place aversion (Jackson et al., 2009).

4.3.5.2.1 Chronic nicotine treatment

In order to use the model of CPA, all animals have to be chronically treated. Twenty days before the experiment, all animals underwent surgery and ALZET® 2004 osmotic pumps were implanted under the skin of the back of the mice. These pumps have a pump rate of 25 µl/h and the animals receive 10 mg/kg/day or 36 mg/kg/day of nicotine base. Control animals received saline. Stock solutions were prepared sterile. The ALZET® 2004 osmotic pumps were filled with the different solutions under sterile conditions following the instructions of Charles River. Afterwards, the pumps were "primed" overnight at 37°C in an incubator. Pumps were stored in a small, closed bottle, filled with autoclaved saline.

For implanting of pumps, animals had to be older than 11 weeks and to weigh at least 22 g. It was important to take care that pumps fit under the skin of the mice and the natural behaviour of the mice is not disturbed. Animals were anesthetised with fully antagonised anaesthesia (FAA or sleep mix), the skin opened with a small cut and a pocket formed between the cutis and subcutis. Afterwards, the pumps were fit into the pocket and the skin closed with 2-3 stitches. Animals were woken up with the antagonistic mix and received short time analgesia with Novaminsulfon.

4.3.5.2.2 Induction of aversion

After chronic nicotine treatment, withdrawal was induced by a single i.p. injection of mecamylamine hydrochloride (3.5 mg/kg), a non-competitive nicotinic acetylcholine receptor antagonist which preferentially blocks nicotinic receptors at autonomic ganglia and crosses the blood-brain barrier. This injection was coupled to the naturally preferred box of the CPA test. The blockade of the receptors caused withdrawal effects of nicotine and therefore aversion in the animals.

4.3.6 Model of excitotoxic seizures

Excessive excitatory activity leads to neuronal damage and death, induced by a mechanism known as excitotoxicity (Ben-Ari and Cossart, 2000). The spreading of uncontrolled glutamatergic neurotransmission causes seizures.

The neuroexcitatory kainic acid was weighed and dissolved in 0.9% saline always freshly before experiment. By i.p. injection of the kainic acid (30 mg/kg) in a volume of 10 ml/kg body weight, epileptiform seizures were induced. The intensity of these seizures were monitored by a trained observer blind to the genotype of the mice for 2 hours and scored every 15 min according to the following scale (Marsicano et al., 2003; Schauwecker and Steward, 1997): 0—no response; 1—immobility and staring; 2—forelimb and/or tail extension, rigid posture; 3—repetitive movements, head bobbing; 4—rearing and falling; 5—continuous rearing and falling: 6—severe clonic-tonic seizures; 7—death.

4.4. Statistical analyses

The data was analysed by Student t-test (with Welsh correction as appropriate), Kruskal-Wallis statistic or univariate analyses of variance (ANOVA), as appropriate. Whenever more than two groups had to be compared, ANOVA was followed by Bonferroni post hoc analyses. Additionally, if an interaction between factors was detected in the two way ANOVA, post hoc analyses were performed with a Bonferroni test. A Mann-Whitney-test was used for the analyses of the kainic acid experiment.

5. Results

5.1. Behavioural analyses with conditional CB1 receptor mutant mouse lines

5.1.1. Characterization of basic functions

5.1.1.1 Measurement of body weight

Before beginning with the actual behavioural experiments, body weight of each animal batch was measured. In Fig. 21 the results for one typical $CB1^{f/f;D1\text{-}Cre}$ mouse batch is shown. $CB1^{f/f;D1\text{-}Cre}$ mice do not differ from their respective wild-type littermates (T_{18}=1.273, p=0.2192). Naïve $CB1^{f/f;D1\text{-}Cre}$ animals showed a weight of 30.60 ± 1.127 g whereas their wild-type littermates had 28.70 ± 0.9781 g.

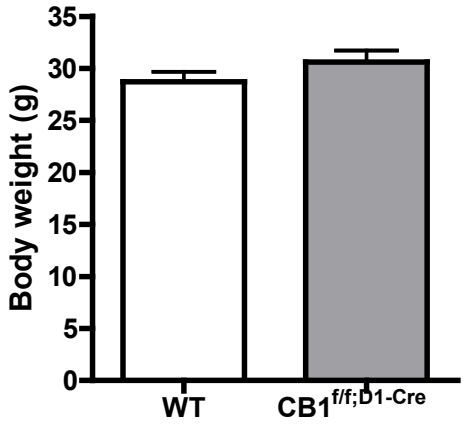

Fig. 21: Body weight of naïve $CB1^{f/f;D1\text{-}Cre}$ and wild-type littermate controls (WT). No significant differences were observed between the groups (t test; n=10/group; male animals; four months old).

5.1.2. Models of locomotion and motor functions

5.1.2.1 Open field test

In the first experiments, I aimed at analysing the basal locomotion of naïve wild-type and $CB1^{f/f;D1\text{-}Cre}$ mice in an open field box for a period of 30 min. The wild-type animals moved a total distance of 6934 ± 319.6 cm, while the $CB1^{f/f;D1\text{-}Cre}$ mice moved a total distance of 7770 ± 417.5 cm. As shown in Fig. 22, no significant difference in the distance moved (T_{18}=1.591, p=0.1291) and for time spent in centre (T_{18}=0.7912, p = 0.4391) were detected between genotypes.

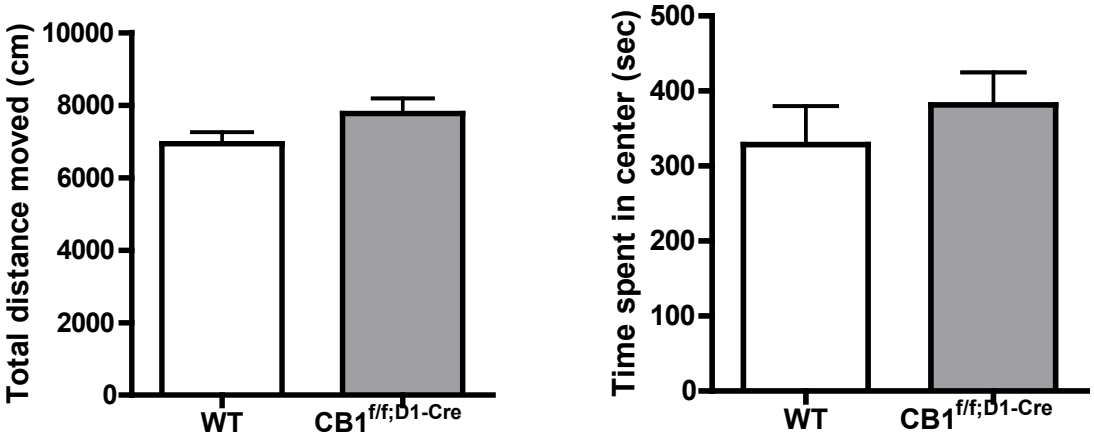

Fig. 22: Basal level in locomotion and time spent in centre of the open field in CB1$^{f/f;D1\text{-}Cre}$ and wild-type littermate controls (WT). No significant differences in distance moved and time spent in centre were detected between the two genotypes (t test; n=10/group; male animals; four months old).

Additionally, I tested the basal locomotion of another batch of CB1$^{f/f;D1\text{-}Cre}$ animals and their respective wild-type littermates over a period of 10 min. During this time the wild-type animals moved a total distance of 4456 ± 90.63 cm, while the CB1$^{f/f;D1\text{-}Cre}$ mice moved a total distance of 4368 ± 131.2 cm. As shown in Fig. 23, no significant difference in the distance moved (T_{21}=0.5618, p=0.1291) and for time spent in centre (T_{21}=1.095, p = 0.2860) were detected between genotypes. CB1$^{f/f;D1\text{-}Cre}$ animals stayed 716.2 ± 86.76 sec in the centre of the open field compared to 611.2 ± 46.15 sec of their wild-type littermates.

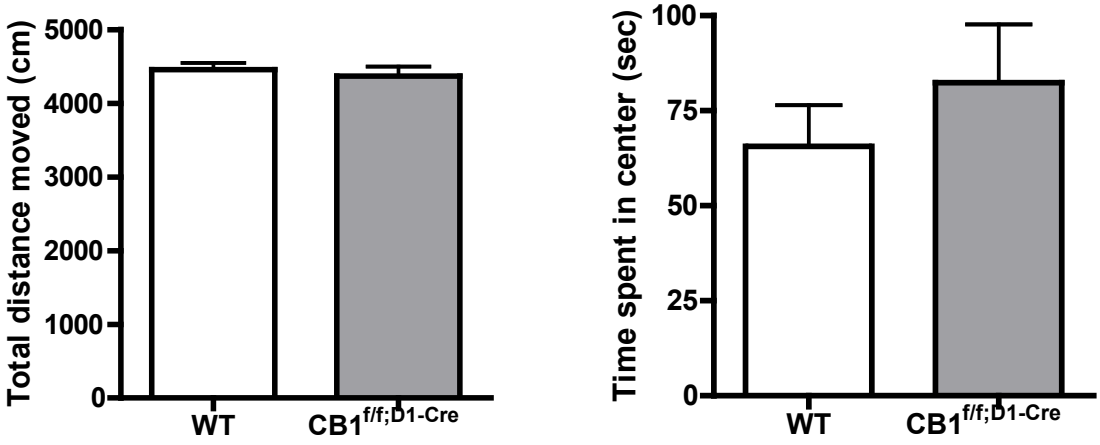

Fig. 23: Basal level in locomotion and time spent in centre of the open field in CB1$^{f/f;D1\text{-}Cre}$ and wild-type littermate controls (WT). No significant differences in distance moved and time spent in centre were detected between the two genotypes (t test; n=12/WT and 11/ CB1$^{f/f;D1\text{-}Cre}$ group; male animals; 6-7 months old).

5.1.2.2 Rotarod test

CB1$^{f/f;D1\text{-}Cre}$ mice and their wild-type littermates were tested in differences in performance and time walking on the rotarod at an age of 4 - 5.5 months. Results of this experiment can be seen in Fig. 24 and 25. Both genotypes were able to increase their time spent on the rod, but only the WT mice (T_{16}=2.619, p=0.0186) and not the CB1$^{f/f;D1\text{-}Cre}$ animals (T_{18}=1.771, p=0.0935) showed a significant increase in time on the rod between session 1 and 5. No significant differences could be found between both genotypes (T_8=0.4789, p= 0.6448).

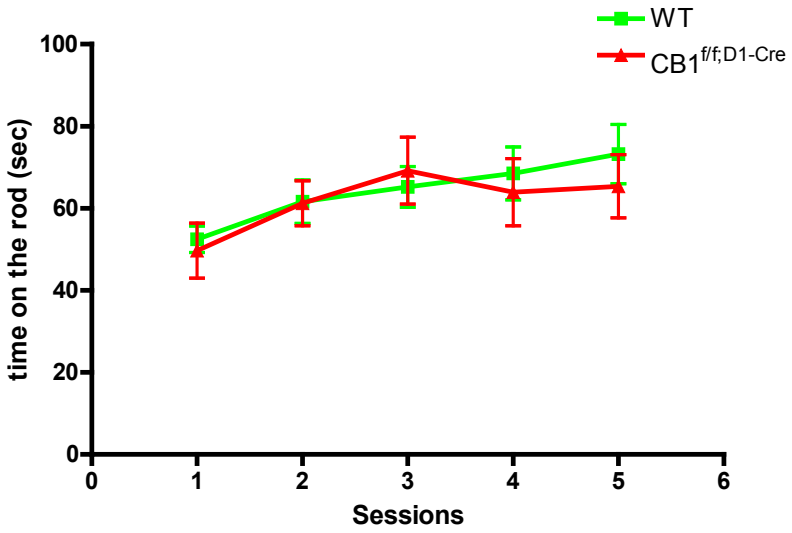

Fig. 24: Time spent walking on the rotarod shown by CB1$^{f/f;D1\text{-}Cre}$ mice compared to their WT littermates. Both groups increased their time spent walking over repeated sessions (n=10/ CB1$^{f/f;D1\text{-}Cre}$ and 9/WT; male animals, 4-5.5 months old)

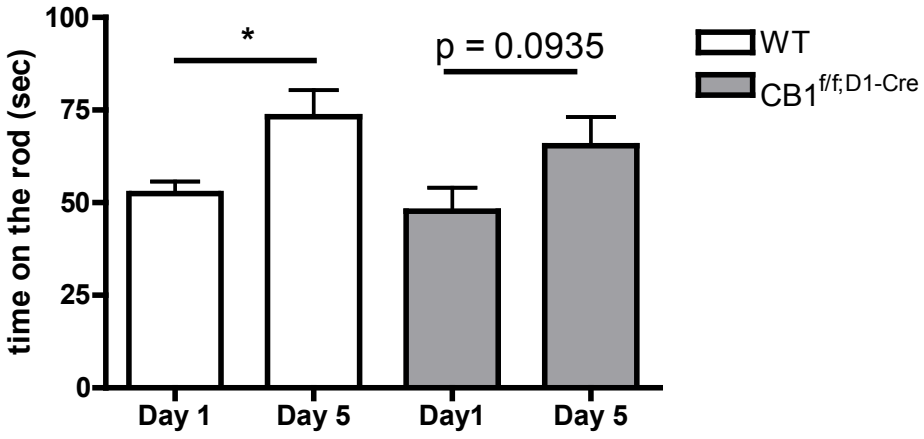

Fig. 25: Differences in time spent on the rod between session day 1 and session day 5 shown by CB1$^{f/f;D1\text{-}Cre}$ mice compared to their WT littermates. WT animals show a significant increase in time on the rod (*p<0.05) (t test; n=10/ CB1$^{f/f;D1\text{-}Cre}$ and 9/WT; male animals, 4-5.5 months old).

5.1.3 Models of emotion

5.1.3.1 Elevated plus maze test

I tested the behaviour of naïve $CB1^{f/f;D1\text{-}Cre}$ mice in different models of emotion. First, anxiety differences between both genotypes in the EPM test were measured (Fig. 26). As one can see in Fig. 25, no differences in the percentage of time spent in open arms (T_{17}=0.5541, p=0.5868) and percentage of entries in open arms (T_{17}=0.3330, p=0.7432) were revealed.

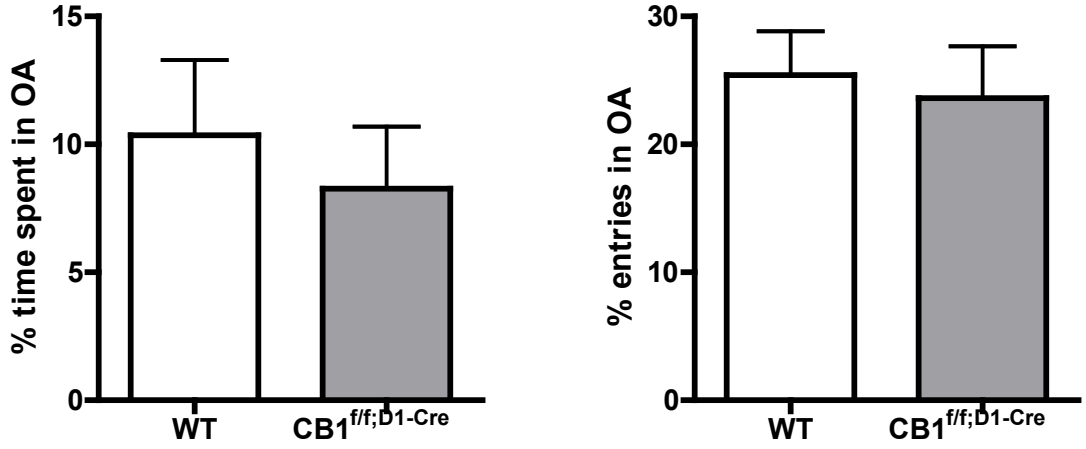

Fig. 26: Basal levels in percentage of time and entries of $CB1^{f/f;D1\text{-}Cre}$ and WT animals in open arms of the elevated plus maze. No difference was detected (t test; n=10/ $CB1^{f/f;D1\text{-}Cre}$ and 9/WT; male animals, 5-7 months old).

Naïve wild-type animals spent 10.36 ± 2.933% of the time in the open arms. This constituted 25.40 ± 3.436% of the entries. The $CB1^{f/f;D1\text{-}Cre}$ mice spent 8.270 ± 2.421% of the time in the open arms that constituted 23.62 ± 4.038% of the entries.

5.1.3.2 Light-dark test

The light-dark-test (LD) is based on the natural conflict of rodents between the tendency exploring unknown areas and the aversion caused by a brightly lit open environment. Here I measured the activity of the animals in exploration of the white compartment. In Fig. 27, the basal behaviour of WT and $CB1^{f/f;D1\text{-}Cre}$ is presented. Neither the results of time spent in the lit compartment (T_{17}=1.364, p=0.1903) nor the results for further entries into the lit compartment (T_{17}=1.315, p=0.2060) differed significantly.

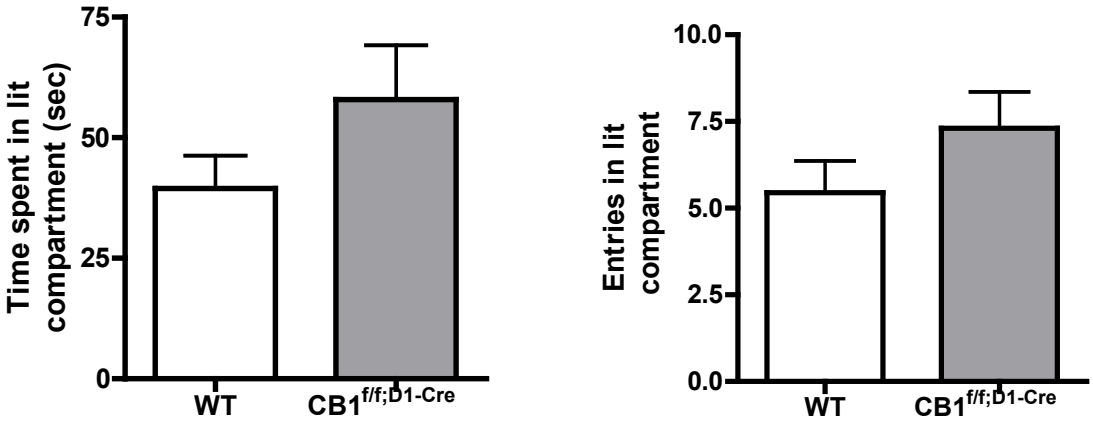

Fig. 27: Basal levels of time spent in and entries into the lit compartment of CB1$^{f/f;D1\text{-}Cre}$ and WT mice. No significant differences were detected (t test; n=10/ CB1$^{f/f;D1\text{-}Cre}$ and 9/WT; male animals, 3-4 months old).

Naïve wild-type mice spent 39.44 ± 6.821 sec in the lit compartment. During this time they made 5.444 ± 0.9146 entries from the dark into the lit compartment. The CB1$^{f/f;D1\text{-}Cre}$ animals spent 57.90 ± 11.25 sec in the lit compartment and showed 7.300 ± 1.055 entries in the lit compartment during the tested time.

5.1.4 Models of recognition memory and learning

All experiments of this part of the thesis were done together with Martin Häring. Both of us contributed equally in this work with the models of recognition memory and learning.

5.1.4.1 Novel object recognition task

The novel object recognition task (NORT) is used to evaluate recognition memory in rodent models. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects a learning and recognition process.

Fig. 28 shows the total time spent in exploration during training period of CB1$^{f/f;D1\text{-}Cre}$ mice. Both genotypes showed no differences in exploration and no natural preference for one or the other object. No significant interaction between the genotype and objects could be observed ($F_{1,44}$=0.50; p=0.4819). Additionally, no further significance is revealed, neither in the effect of genotype ($F_{1,44}$=1.76; p=0.1915) nor in the effect of objects ($F_{1,44}$=0.00; p=0.9618).

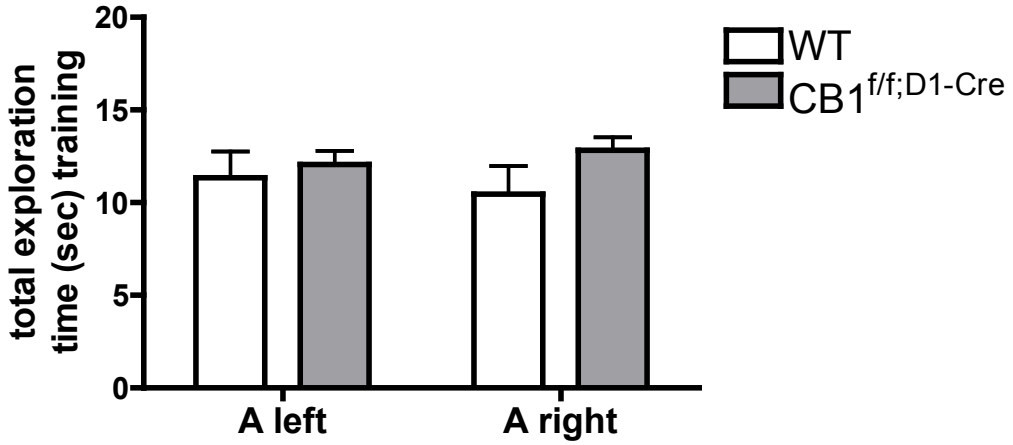

Fig. 28: Total time spent in exploration of both A objects in CB1$^{f/f;D1\text{-}Cre}$ compared to WT animals. No significant differences could be seen between genotypes. (2-WAY ANOVA followed by Bonferroni test; n=12/ group; male animals, 6-7 months old).

After two hours of retention both genotypes show a significant effect in exploration ($F_{1,44}$=4.10; p=0.0495) of the new object (Fig. 29). However, I found no genotype-related differences ($F_{1,44}$=0.75; p=0.3911).

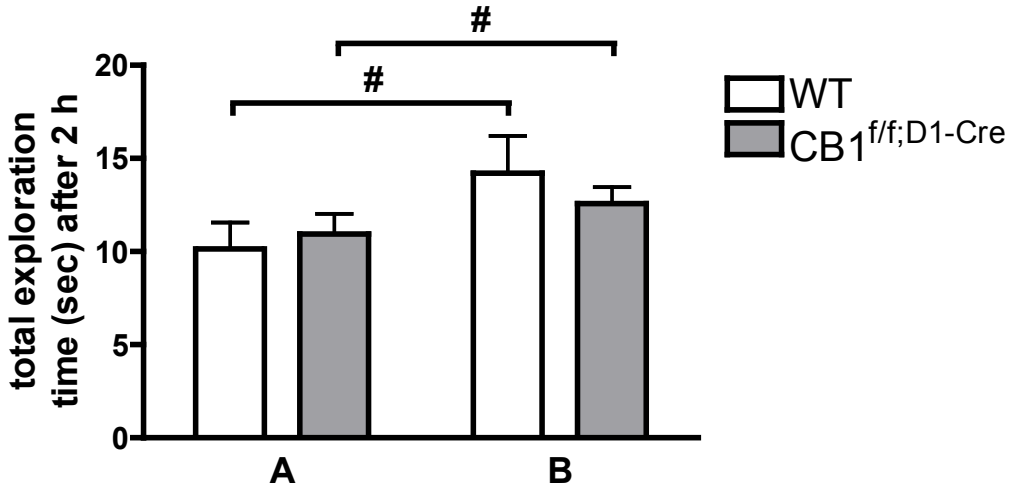

Fig. 29: Total time spent on exploration after two hours of retention in CB1$^{f/f;D1\text{-}Cre}$ and WT animals. Object B was significantly more explored in both groups. $^{\#}p<0.05$ compared to object A (2-WAY ANOVA followed by Bonferroni test; n=12/ group; male animals, 6-7 months old).

The same exploration effect is seen after 24 hours of retention as represented in Fig. 30. Both genotypes showed a significant increase of exploration of object C ($F_{1,44}$=8.97; p=0.0045) compared to object A. But again, no genotype-related differences were seen ($F_{1,44}$=3.32; p=0.0754).

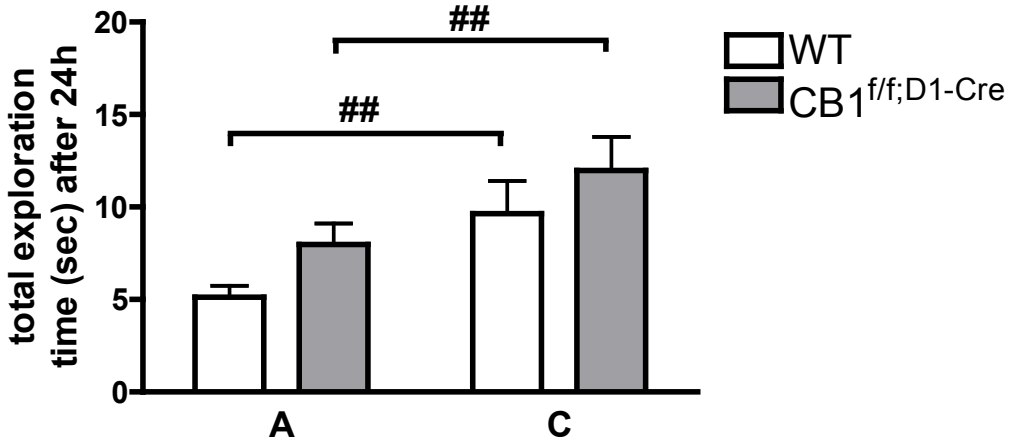

Fig. 30: Total time spent on object exploration after 24 hours of retention in CB1$^{f/f;D1\text{-}Cre}$ mice and their WT littermates. Object C was significantly more explored in both groups. $^{\#\#}p<0.01$ compared to object A (2-WAY ANOVA followed by Bonferroni test; n=12/ group; male animals, 6-7 months old).

Next I tested two additional mutant mouse lines, the CB1$^{f/f;dlx5/6\text{-}Cre}$ and CB1$^{f/f;NEX\text{-}Cre}$ mice in the NORT. Fig. 31 shows the results of total exploration time of CB1$^{f/f;dlx5/6\text{-}Cre}$ and CB1$^{f/f;NEX\text{-}Cre}$ mice in the training session. I saw no natural preference for one of the objects in CB1$^{f/f;dlx5/6\text{-}Cre}$ animals and their wild type littermates ($F_{1,74}$=0.45; p=0.5037). On the other hand, CB1$^{f/f;dlx5/6\text{-}Cre}$ animals, spent significantly more time exploring the objects than their wild type littermates ($F_{1,74}$=17.88; p<0.0001).

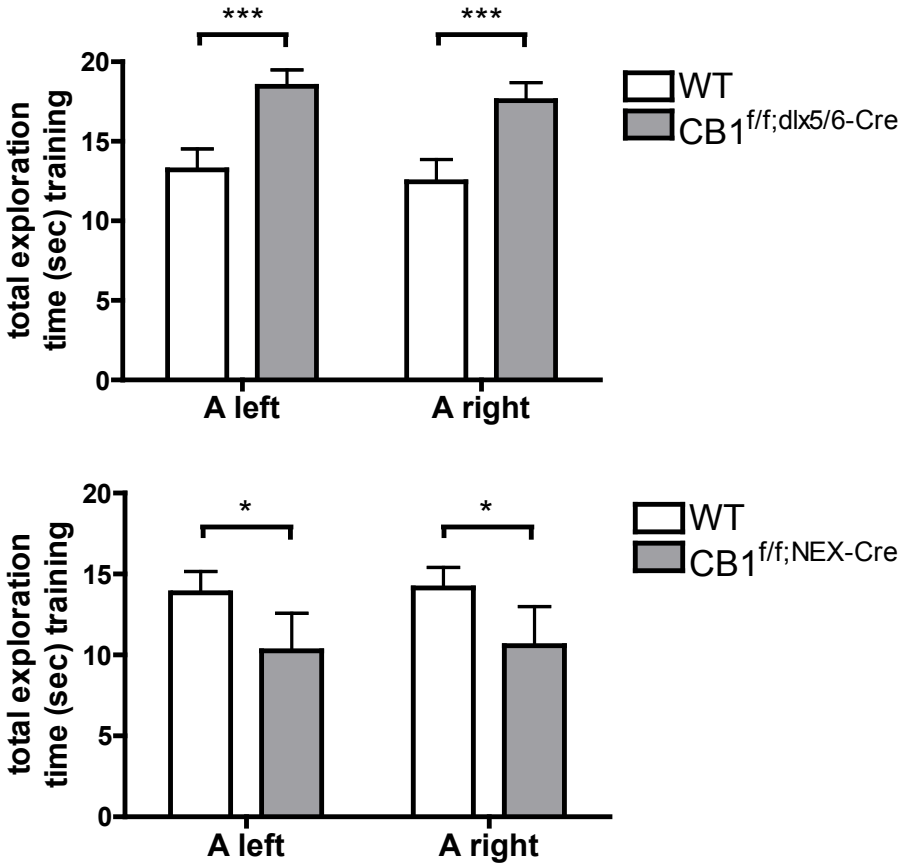

Fig. 31: Total time spent on exploration of both A objects in CB1$^{f/f;dlx5/6\text{-}Cre}$ (upper panel) and CB1$^{f/f;NEX\text{-}Cre}$ mice (lower panel) compared to their WT littermates. Object A was equally explored in both mouse lines. Significant differences in basal exploration are shown for genotypes. *p<0.05 and ***p<0.0001 compared to respective wild-types (2-WAY ANOVA followed by Bonferroni test; CB1$^{f/f;dlx5/6\text{-}Cre}$ n=16/WT and n=23/KO; male animals, 5-6.5 months old; CB1$^{f/f;NEX\text{-}Cre}$ n=22/WT and n=13/KO; male animals, 5-9 months old).

In CB1$^{f/f;NEX\text{-}Cre}$ mice an opposite phenotype was observed: these animals explored objects significantly less than their wild type littermates ($F_{1,62}$=4.18; p=0.0451). However, CB1$^{f/f;NEX\text{-}Cre}$ mice showed no dissimilarity in exploring the left or the right object A in the training session ($F_{1,62}$=0.03; p=0.8564).

After two hours of retention the results of total exploration time of both mouse lines are shown in Fig 32. CB1$^{f/f;dlx5/6\text{-}Cre}$ mice spent significantly more time exploring both objects than their wild-type littermates ($F_{1,74}$=8.49; p=0.0049). Interestingly, however, I saw no significant difference in the time spent exploring the two objects (familiar vs. new) in either of the genotypes ($F_{1,74}$=0.00; p=0.9938). In contrast, CB1$^{f/f;NEX\text{-}Cre}$ mice spent significantly less time exploring both objects than their wild-type littermates ($F_{1,66}$=13.68; p=0.0004). At the same time, wild-type mice spent significantly more time exploring the unfamiliar object than the

familiar one (T_{42}=2.674; p=0.0106) while this phenomenon was not detectable in the mutants (T_{24}=0.6268; p= 0.5367).

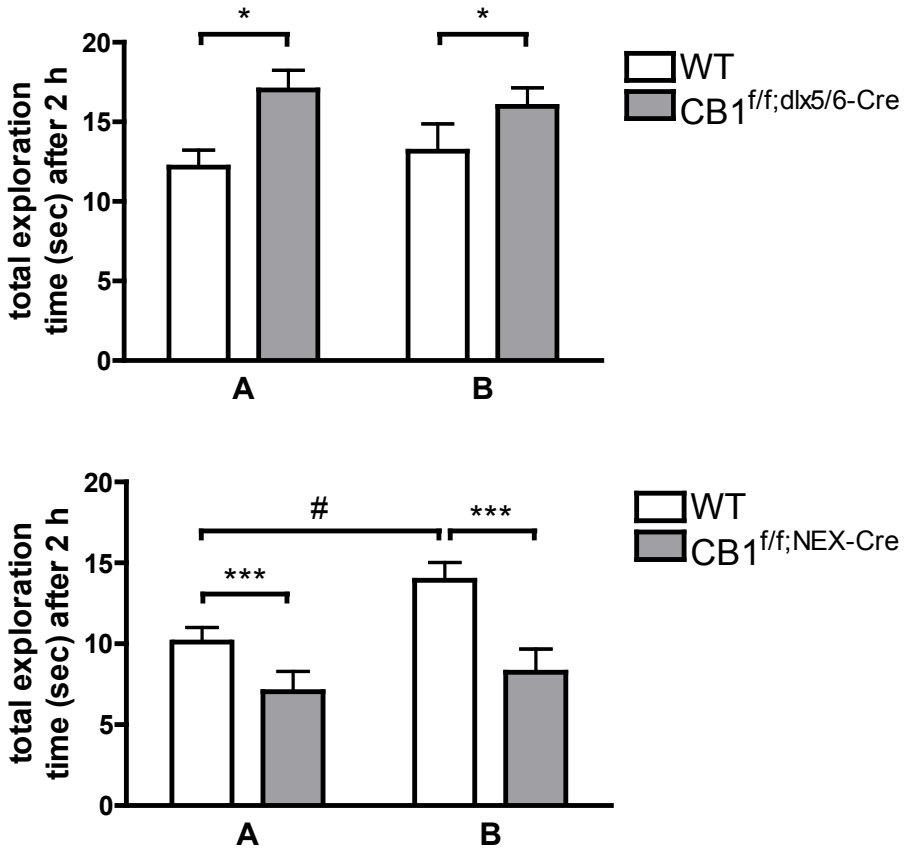

Fig. 32: Total time spent on exploration of object A and B in CB1$^{f/f;dlx5/6\text{-}Cre}$ (upper panel) and CB1$^{f/f;NEX\text{-}Cre}$ mice (lower panel) compared to their WT littermates after two hours of retention. Object A was equally explored in both mouse lines. Significant differences in basal exploration are shown for genotypes. *p<0.05 and ***p<0.0001 compared to respective wild-types, (2-WAY ANOVA followed by Bonferroni test; CB1$^{f/f;dlx5/6\text{-}Cre}$ n=16/WT and n=23/KO; male animals, 5-6.5 months old; CB1$^{f/f;NEX\text{-}Cre}$ n=22/WT and n=13/KO; male animals, 5-9 months old), #p<0.05 compared to object A (t test; CB1$^{f/f;NEX\text{-}Cre}$ n=22/WT and n=13/KO; male animals, 5-9 months old)

The graphs in Fig. 33 present the results of total exploration time after 24 hours of retention in CB1$^{f/f;dlx5/6\text{-}Cre}$ and CB1$^{f/f;NEX\text{-}Cre}$ mouse line. Both CB1$^{f/f;dlx5/6\text{-}Cre}$ mice and their wild-type littermates spent significantly more time exploring object C (unfamiliar object) than object A. (T_{44}=2.645; p=0.0113and T_{30}=2.511; p=0.0177 for mutants and wild-types, respectively). At the same time, CB1$^{f/f;dlx5/6\text{-}Cre}$ mice spent significantly more time exploring both objects ($F_{1,74}$=6.17; p=0.0152) than their wild-type littermates. However, no significant interaction between the genotype and objects ($F_{1,74}$=0.08; p=0.7805) could be detected in CB1$^{f/f;dlx5/6\text{-}Cre}$ mice.

Furthermore, CB1$^{f/f;NEX-Cre}$ animals spent significantly more time exploring object C (unfamiliar object) than object A (T_{42}=3983; p=0.0003). This phenomenon could not be detected in the mutants (T_{24}=0.9470; p=0.3531). At the same time, CB1$^{f/f;NEX-Cre}$ mice spent significantly less time exploring both objects ($F_{1,66}$=32.87; p<0.0001) than their wild-type littermates .Further analysis showed a significant interaction between the genotype and objects ($F_{1,66}$=4.25; p=0.0423) demonstrating that memory over 24 hours has a different course of development. in the CB1$^{f/f;NEX-Cre}$ mice than in the wild-type littermates.

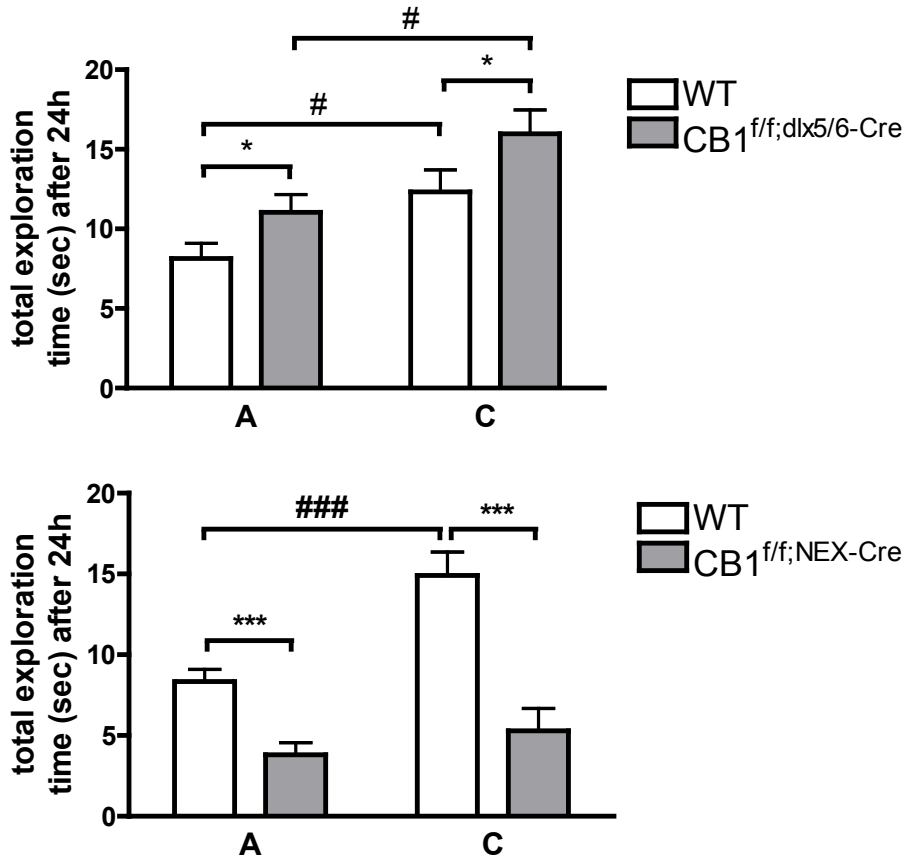

Fig. 33: Total time spent on exploration of object A and C in CB1$^{f/f;dlx5/6-Cre}$ (upper panel) and CB1$^{f/f;NEX-Cre}$ mice (lower panel) compared to their WT littermates after 24 hours of retention. Object A was equally explored in both mouse lines. Significant differences in basal exploration are shown for genotypes. *p<0.05 and ***p<0.0001 compared to respective wild-types, (2-WAY ANOVA followed by Bonferroni test; CB1$^{f/f;dlx5/6-Cre}$ n=16/WT and n=23/KO; male animals, 5-6.5 months old; CB1$^{f/f;NEX-Cre}$ n=22/WT and n=13/KO; male animals, 5-9 months old), #p<0.05 compared to object A, $^{###}$p<0.0001 (t test; CB1$^{f/f;dlx5/6-Cre}$ n=16/WT and n=23/KO; male animals, 5-6.5 months old; CB1$^{f/f;NEX-Cre}$ n=22/WT and n=13/KO; male animals, 5-9 months old)

Evaluation of the discrimination index (DI) revealed that no groups, independent of the line, show differences within the training session regarding the exploration of the left and the right object A [$CB1^{f/f;D1\text{-}Cre}$ knock-out mice (T_{11}=1.447, p=0.1758); $CB1^{f/f;D1\text{-}Cre}$ wild-type mice (T_{11}=1.679, p=0.1213); $CB1^{f/f;dlx5/6\text{-}Cre}$ knock-out animals (T_{15}=1.118, p=0.2812); $CB1^{f/f;dlx5/6\text{-}Cre}$ wild-type littermates (T_{22}=1.959, p=0.0630); $CB1^{f/f;NEX\text{-}Cre}$ knock-out mice (T_{20}=0.8230, p=0.4202); $CB1^{f/f;NEX\text{-}Cre}$ wild-type mice (T_{11}=0.9582, p=0.3585); Tab. 4]. Furthermore, no discrimination differences compared to their respective wild-type controls could be seen for all mouse lines within the training session [$CB1^{f/f;D1\text{-}Cre}$ line (T_{22}=1.951, p=0.0639); $CB1^{f/f;dlx5/6\text{-}Cre}$ line (T_{37}=0.06488, p=0.9486); $CB1^{f/f;NEX\text{-}Cre}$ line (T_{31}=1.407, p=0.1693); Tab. 4].

The results for 2h retention session showed that several groups lacked a significant discrimination between the familiar and the novel object. Only wild-type mice of $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;D1\text{-}Cre}$ line and the $CB1^{f/f;D1\text{-}Cre}$ knock-out animals displayed a significant preference towards the novel stimulus [$CB1^{f/f;D1\text{-}Cre}$ wild-type mice (T_{10}=2.502, p=0.0313); $CB1^{f/f;D1\text{-}Cre}$ knock-out mice (T_{10}=2.238, p=0.0492); $CB1^{f/f;dlx5/6\text{-}Cre}$ wild-type littermates (T_{15}=0.07097, p=0.9444); $CB1^{f/f;dlx5/6\text{-}Cre}$ knock-out mice (T_{22}=1.366, p=0.1858); $CB1^{f/f;NEX\text{-}Cre}$ wild-type (T_{21}=4.806, p<0.0001); $CB1^{f/f;NEX\text{-}Cre}$ knock-out mice (T_{12}=1.220, p=0.2458); Tab. 4]. Comparing the mutants and their respective wild-type littermates did not reveal significant differences in any lines [$CB1^{f/f;D1\text{-}Cre}$ line (T_{20}=0.9965, p=0.3309); $CB1^{f/f;dlx5/6\text{-}Cre}$ (T_{37}=0.6235, p=0.5368); $CB1^{f/f;NEX\text{-}Cre}$ line (T_{33}=1.775, p=0.0850); Tab. 4]. In the 24h retention phase, all groups, independent of the genotype and except of $CB1^{f/f;NEX\text{-}Cre}$ knock-out animals, showed a significant preference towards the novel object C [$CB1^{f/f;NEX\text{-}Cre}$ wild-type animals (T_{21}=4.472, p=0.0002); $CB1^{f/f;NEX\text{-}Cre}$ knock-out mice (T_{12}=0.4328, p=0.6729); $CB1^{f/f;dlx5/6\text{-}Cre}$ wild-type mice (T_{15}=2.818, p=0.0129); $CB1^{f/f;dlx5/6\text{-}Cre}$ knock-out animals (T_{22}=3.072, p=0.0056); $CB1^{f/f;D1\text{-}Cre}$ wild-type mice (T_{11}=3.601, p=0.0042); $CB1^{f/f;D1\text{-}Cre}$ knock-out animals (T_{11}=3.540, p=0.0046); Tab. 4]. Comparison between the mutants and their respective wild-type littermates displayed no genotype difference [$CB1^{f/f;D1\text{-}Cre}$ line (T_{22}=1.049, p=0.3055); $CB1^{f/f;dlx5/6\text{-}Cre}$ line T_{37}=0.1255, p=0.9008; $CB1^{f/f;NEX\text{-}Cre}$ line (T_{33}=1.522, p=0.1374); Tab. 4].

	CB1$^{f/f;D1\text{-}Cre}$		CB1$^{f/f;dlx5/6\text{-}Cre}$		CB1$^{f/f;NEX\text{-}Cre}$	
Paradigm	**+/+**	**-/-**	**+/+**	**-/-**	**+/+**	**-/-**
Distance moved (cm)						
Open Field	4456 ± 91	4368 ± 131	2621 ± 306	3801 ± 202**	2824 ± 209	2324 ± 182
Sociability test						
Habituation Phase	1669 ± 81	1850 ± 118	1597 ± 59	1730 ± 64	2012 ± 113	1569 ± 162 *
Sociability Phase	5055 ± 162	5079 ± 157	4945 ± 127	5083 ± 175	5171 ± 205	4891 ± 312
Social Novelty Phase	3863 ± 292	3942 ± 160	4023 ± 141	4623 ± 175 *	3973 ± 211	3125 ± 227 *
Time in centre (sec)						
Open Field	65.4 ± 11	82.4 ± 15	156.0 ± 48	82.0 ± 21	63.3 ± 12	80.9 ± 20
Discrimination index (DI)						
NORT						
Training Session	-0.08 ± 0.05	0.03 ± 0.02	-0.03 ± 0.03	-0.03 ± 0.02	0.01 ± 0.01	-0.08 ± 0.08
Retention Session 2h	0.15 ± 0.06#	0.08 ± 0.03#	0.00 ± 0.06	-0.03 ± 0.03	0.16 ± 0.03#	0.06 ± 0.05
Retention Session 24h	0.27 ± 0.08#	0.18 ± 0.05#	0.18 ± 0.06#	0.15 ± 0.05#	0.25 ± 0.06#	0.06 ± 0.13
Sociability test						
Sociability Phase	0.20 ± 0.04#	0.30 ± 0.04#	0.27 ± 0.03#	0.35 ± 0.04#	0.29 ± 0.03#	0.12 ± 0.07*
Social Novelty Phase	0.03 ± 0.06	0.03 ± 0.06	-0.01 ± 0.05	0.09 ± 0.03#	0.05 ± 0.03	0.08 ± 0.09

Table 4: Locomotion, anxiety and memory. Evaluation of locomotion (distance moved), anxiety (time in centre) and memory (discrimination index) for CB1$^{f/f;D1\text{-}Cre}$, CB1$^{f/f;dlx5/6\text{-}Cre}$ and CB1$^{f/f;NEX\text{-}Cre}$ lines; +/+ (wild-type), -/- (knock-out); t-test analysis: *p<0.05; **p<0.01 (significance between genotype); #p<0.05 (significant from 0; positive recognition of novel object).

The evaluation of object specific exploration (A left or A, B, C right) over the three session (training, 2h retention and 24h retention), revealed a significant difference for the CB1$^{f/f;NEX\text{-}Cre}$ knock-out animals compared to their littermate controls. Thus, the CB1$^{f/f;NEX\text{-}Cre}$ mutants showed a steadily decreasing investigatory behaviour for both the left object (increasing familiarity) and the right object (always novel) (CB1$^{f/f;NEX\text{-}Cre}$ knock-out animals: interaction (object versus time): $F_{(2,48)}$=0.1537; p=0.8580; Bonferroni post hoc analysis: training session: p>0.05 (Fig. 29 right panel), 2h retention session: p>0.05 (Fig 30 right panel), 24h retention session: p>0.05 (Fig. 31 right panel)). Differently, this effect can be seen in the wild-type mice of CB1$^{f/f;NEX\text{-}Cre}$ line only for the left objects (increasing familiarity), while the time spent investigating the right objects (always novel) remained constant [CB1$^{f/f;NEX\text{-}Cre}$ wild-type animals: interaction (object versus time): $F_{(2,84)}$=4.851, p=0.0101; Bonferroni post hoc analysis: training session: p>0.05 (Fig. 30 right panel), 2h retention session: p>0.05 (Fig 31 right pan-

el), 24h retention session: p<0.01 (Fig. 32 right panel)]. Additionally, it was possible to detect a significant difference between the genotypes in exploring the right side objects but not the left side objects over the three sessions [left object interaction (genotype versus time): $F_{(2,48)}$=0.2283, p=0.7965; Bonferroni post hoc analysis: training session: p>0.05 (Fig. 30 right panel), 2h retention session: p>0.05 (Fig. 31 right panel), 24h retention session: p>0.05 (Fig. 32 right panel); right object interaction (genotype versus time): $F_{(2,66)}$=3.522, p=0.0352; Bonferroni post hoc analysis: training session: p>0.05 (Fig. 30 right panel), 2h retention session: p>0.05 (Fig.31 right panel), 24h retention session: p<0.001 (Fig. 32 right panel)].

5.1.4.2 Sociability test

I used a modified sociability test, which was based on the protocol of Moy et al. (2004) in order to detect differences between $CB1^{f/f;D1\text{-}Cre}$, $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ mouse lines and to reveal genotype differences within each line.

The analysis of the $CB1^{f/f;D1\text{-}Cre}$ animals did not reveal any significant genotype differences in the three different phases of the sociability test (Fig. 34, 35). Only a non-significant trend was observed in the sociability phase. Even though time spent in the middle area was not altered (T_{28}=0.9190; p=0.3659), it seems that the mutant showed a slight preference to explore the interaction partner (T_{28}=1.909; p=0.0666) rather than the empty cage (T_{28}=1.859; p=0.0736), i.e. the object (Fig. 34). In the social novelty phase, not even a trend was detectable (familiar T_{30}=0.7636; p=0.4511; novel T_{30}=0.5840; p=0.5636; middle T_{30}=0.6112; p=0.5457; Fig. 35).

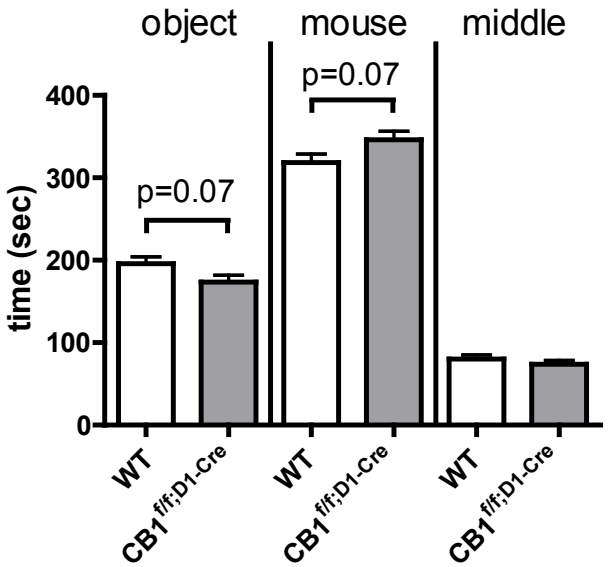

Fig. 34 Animate vs. inanimate exploration in $CB1^{f/f;D1\text{-}Cre}$ compared to WT littermates during the sociability phase. No genotype differences were observed (t test; n=16/WT and n=14/KO; male animals, 6-7 months old).

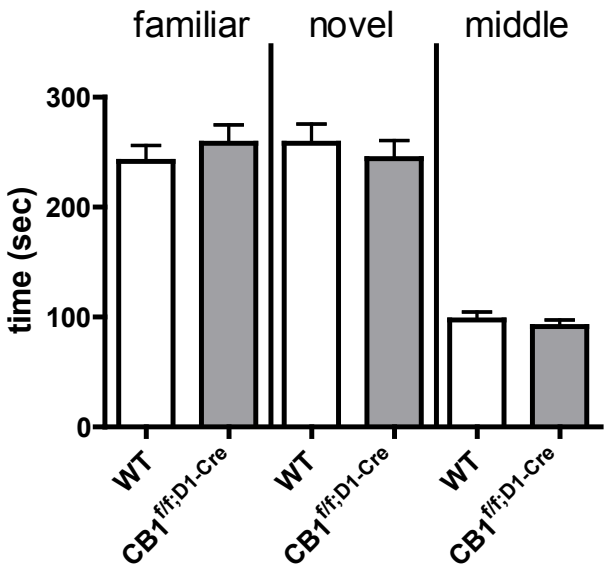

Fig. 35: Exploration of the familiar and the novel interaction partner in CB1$^{f/f;D1\text{-}Cre}$ compared to WT littermates during the social novelty phase. No genotype differences were observed (t test; n=17/WT and n=15/KO; male animals, 6-7 months old).

As presented in Fig. 36 the CB1$^{f/f;dlx5/6\text{-}Cre}$ mice showed a significant increase in time spent with the novel interaction partner as compared to littermates as controls (sociability T_{57}=2.099; p=0.0403; social novelty T_{35}=3.063; p=0.0042) in the sociability phase as well as in the social novelty phase. The time spent in the middle compartment was consequently decreased (sociability T_{57}=2.740; p=0.0082; social novelty T_{35}=2.168, p=0.037). Interestingly, the time spent in the compartment with the empty cage (i.e. the object only) during the sociability phase as well as the time spent with the familiar animal (social novelty test) were not different between CB1$^{f/f;dlx5/6\text{-}Cre}$ animals and their wild-type littermates (object T_{57}=1.114; p=0.2699; familiar T_{35}=1.017; p=0.3162).

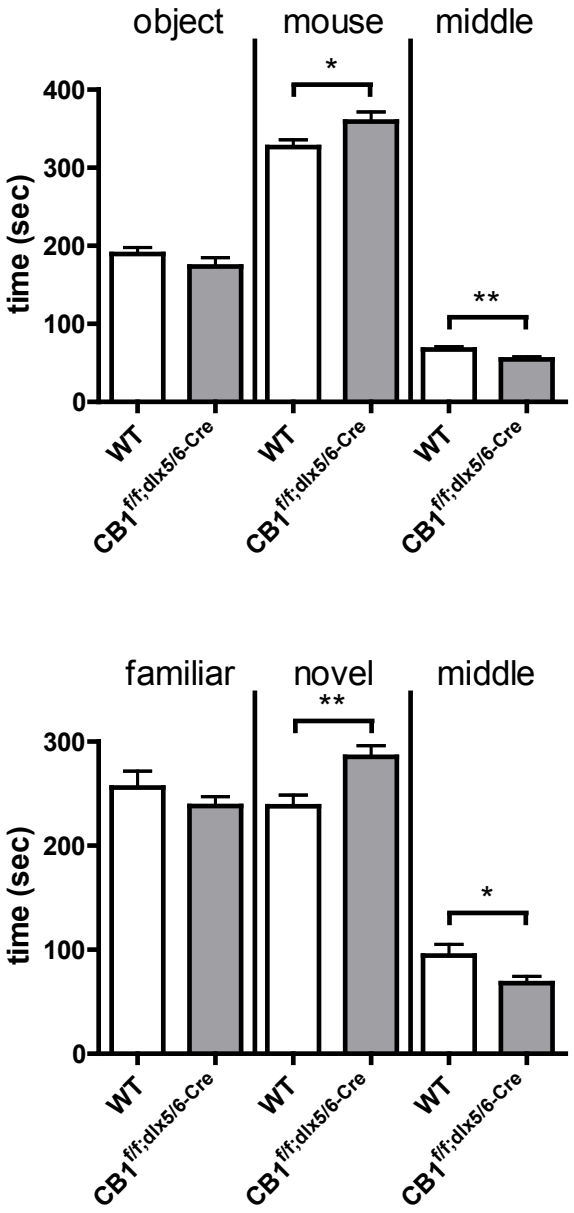

Fig. 36: Animate vs. inanimate exploration (upper panel) and exploration of the familiar and the novel interaction partner (lower panel) in $CB1^{f/f;dlx5/6\text{-}Cre}$ compared to WT littermates during the sociability and social novelty phase. $CB1^{f/f;dlx5/6\text{-}Cre}$ mice showed an increased social interaction in both sessions *p<0.05, **p<0.01 (t test; sociability n=28/WT and n=31/KO; social novelty n=17/WT and n=20/KO; male animals, 5-6.5 months old).

In contrast to the findings with $CB1^{f/f;dlx5/6\text{-}Cre}$ line, the $CB1^{f/f;NEX\text{-}Cre}$ animals showed a significant increase in time spent in the middle compartment (T_{33}=2.247; p=0.0314; Fig. 37) during the sociability phase. Accordingly, these mutants displayed a significant decrease in time spent with the interaction partner but not with the object (mouse T_{33}=3.734; p= 0.0007; object T_{33}=1.412; p=0.1672). A similar result is obtained when the novel interaction partner was

introduced during the social novelty test. While the CB1$^{f/f;NEX\text{-}Cre}$ mice spend more time in the middle compartment, they spent less time with the familiar and novel partner compared to their wild-type littermates (middle T_{33}=3.772; p=0.006; familiar T_{33}=2.263; p=0.0303; unknown T_{33}=2.596, p=0.0140).

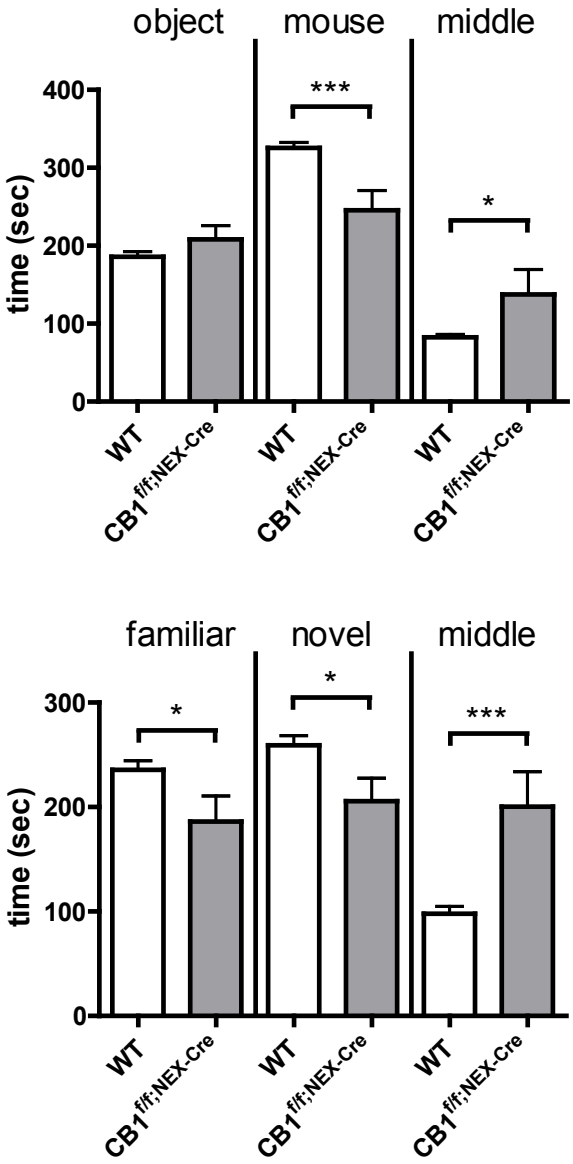

Fig. 37: Animate vs. inanimate exploration (upper panel) and exploration of the familiar and the novel interaction partner (lower panel) in CB1$^{f/f;NEX\text{-}Cre}$ mice compared to WT littermates during the social novelty phase. CB1$^{f/f;NEX\text{-}Cre}$ mice displayed no significant change in the exploration session, where there is a choice between the object and the interaction partner. In the social novelty phase the interaction with a novel interaction partner is decreased compared to WT controls.*p<0.05, ***p<0.001 (t test; n=22/WT and n=13/KO; male animals, 5-9 months old)

For this test, I evaluated the DI as well. But only minimal differences between the genotypes could be found. In the sociability phase, the $CB1^{f/f;NEX\text{-}Cre}$ animals showed an impaired preference towards the interaction partner compared to their respective littermates (T_{33}=2.537, p=0.0161; Tab. 4). In contrast, the $CB1^{f/f;D1\text{-}Cre}$ knock-out mice and the $CB1^{f/f;dlx5/6\text{-}Cre}$ knock-out mice showed no significant changes in the preference towards the interaction partner [$CB1^{f/f;D1\text{-}Cre}$ knock-out mice (T_{28}=1.636, p=0.1130); $CB1^{f/f;dlx5/6\text{-}Cre}$ knock-out mice (T_{57}=1.507, p=0.1373); Tab. 4]. In the social novelty phase, no DI differences were observed in any of the lines [$CB1^{f/f;D1\text{-}Cre}$ line (T_{30}=0.6126 p= 0.547); $CB1^{f/f;dlx5/6\text{-}Cre}$ line (T_{34}=1.794, p=0.0817); $CB1^{f/f;NEX\text{-}Cre}$ line (T_{33}=0.3977, p=0.6934); Tab. 4].

5.1.4.3 Evaluation of locomotor activity in novel object recognition task and sociability test

The evaluation of the locomotor activity in the habituation phase of NORT equates a usual open field. This evaluation revealed that only $CB1^{f/f;dlx5/6\text{-}Cre}$ knock-out mice differed (T_{18}=3.213, p=0.0048; Tab. 4) from their respective wild-types. None of the other mouse lines displayed a change in the distance moved compared with their respective wild-type littermates in the open field [$CB1^{f/f;D1\text{-}Cre}$ line (T_{21}=0.5618, p=0.5802); $CB1^{f/f;NEX\text{-}Cre}$ line (T_{34}=1.609, p=0.1169); Tab. 4]. In regard to the time spent in the centre of open field, I was not able to detect an alteration in any of the conditional mutant lines [$CB1^{f/f;D1\text{-}Cre}$ line (T_{21}= 0.9048, p 0.3758); $CB1^{f/f;dlx5/6\text{-}Cre}$ line (T_{18}=1.418, p=0.1733); $CB1^{f/f;NEX\text{-}Cre}$ line (T_{34}=0.8168, p=0.4197); Tab. 4].

Additionally, I measured the locomotor activity in the habituation phase of the sociability test. The results did not reveal significant changes except for the $CB1^{f/f;NEX\text{-}Cre}$ knock-out mice, which showed a decrease in locomotion [$CB1^{f/f;NEX\text{-}Cre}$ line (T_{33}=2.312, p=0.0271); $CB1^{f/f;D1\text{-}Cre}$ line T_{29}=1.571, p=0.1270); $CB1^{f/f;dlx5/6\text{-}Cre}$ line (T_{60}=1.506, p=0.1374); Tab. 4]. In the sociability phase, no alteration in the distance moved was observed in any of the lines [$CB1^{f/f;D1\text{-}Cre}$ line (T_{30}=0.1082, p=0.9145); $CB1^{f/f;dlx5/6\text{-}Cre}$ line (T_{62}=0.6159, p=0.5402); $CB1^{f/f;NEX\text{-}Cre}$ line (T_{29}=0.7833, p=0.4398); Tab. 4]. In the social novelty phase, no change in the distance moved was found for $CB1^{f/f;D1\text{-}Cre}$ mice compared to their respective wild-type littermates (T_{30}=0.2386, p=0.8130). But I was able to detect a significant decrease in the distance moved for the $CB1^{f/f;NEX\text{-}Cre}$ mice compared to wild-type littermates $CB1^{f/f;NEX\text{-}Cre}$ line (T_{33}=2.575, p=0.0146). Additionally, a significant increase in the distance moved was measured for the $CB1^{f/f;dlx5/6\text{-}Cre}$ line (T_{38}=2.591, p=0.0135) as presented in Tab. 4.

5.1.4.4 Resident-intruder test

The resident-intruder test was performed by placing a novel, group-housed "intruder" mouse (C57BL/6N) into the home cage of the single-housed animal ("resident"; $CB1^{f/f;D1\text{-}Cre}$, $CB1^{f/f;dlx5/6\text{-}Cre}$ or $CB1^{f/f;NEX\text{-}Cre}$ mouse).

As it can be seen in the following figures, the analysis of the $CB1^{f/f;D1\text{-}Cre}$ animals did not display any significant genotype differences in this paradigm, neither in interaction time spent with the intruder (T_{20}=0.3481; p=0.7314; Fig. 38) nor in fighting behaviour compared to their wild-type littermates (fights T_{22}=0.0000, p=1.0; fighting time T_{22}=0.8261, p=0.4176; Fig.39).

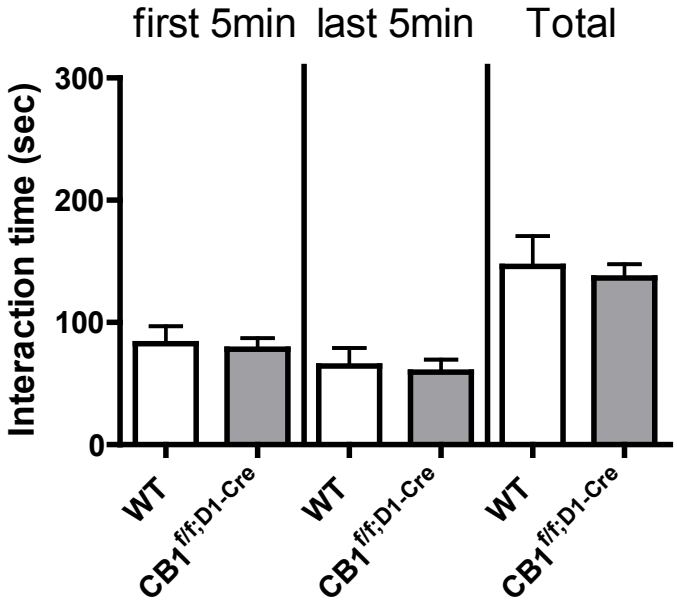

Fig. 38: Animate exploration in the resident-intruder test. Social interaction with an unknown, younger intruder for $CB1^{f/f;D1\text{-}Cre}$ compared to respective WT littermates. No genotype differences were observed (t test; n=11/WT and n=11/KO; male animals, 6-7 months old).

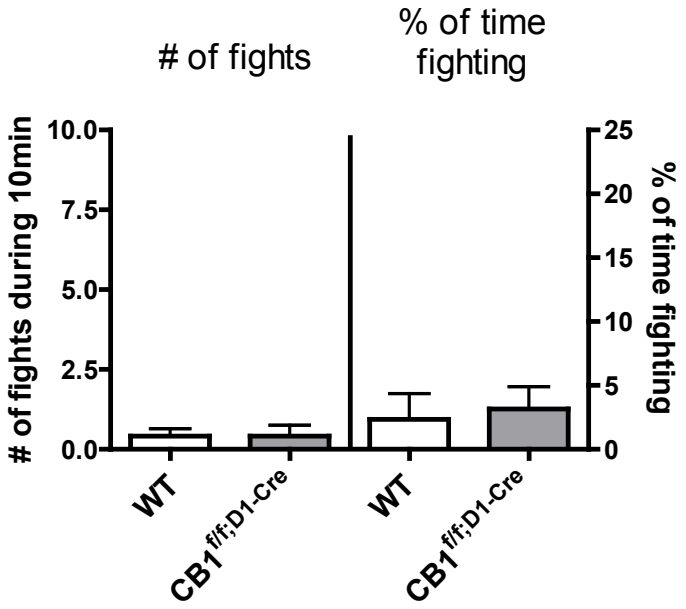

Fig. 39: Animate exploration in the resident-intruder test. Number of fights induced by the resident and percentage of time fighting in $CB1^{f/f;D1\text{-}Cre}$ animals. Mutants showed no behavioural changes as compared to their wild-type control littermates (t test; n=12/WT and n=12/KO; male animals, 6-7 months old).

Results detected in the $CB1^{f/f;dlx5/6\text{-}Cre}$ animals are presented in Fig 40 (left). Mutants showed an increased interaction with the intruder animal (T_{27}=2.522; p=0.0174) compared to their wild-type littermates. As it can be seen in the right panel of Fig. 40, the overall fighting with the younger intruder did not change as compared with the wild-type littermates (fights T_{26}=0.4227, p= 0.6760; fighting time T_{29}=0.6286; p=0.5345).

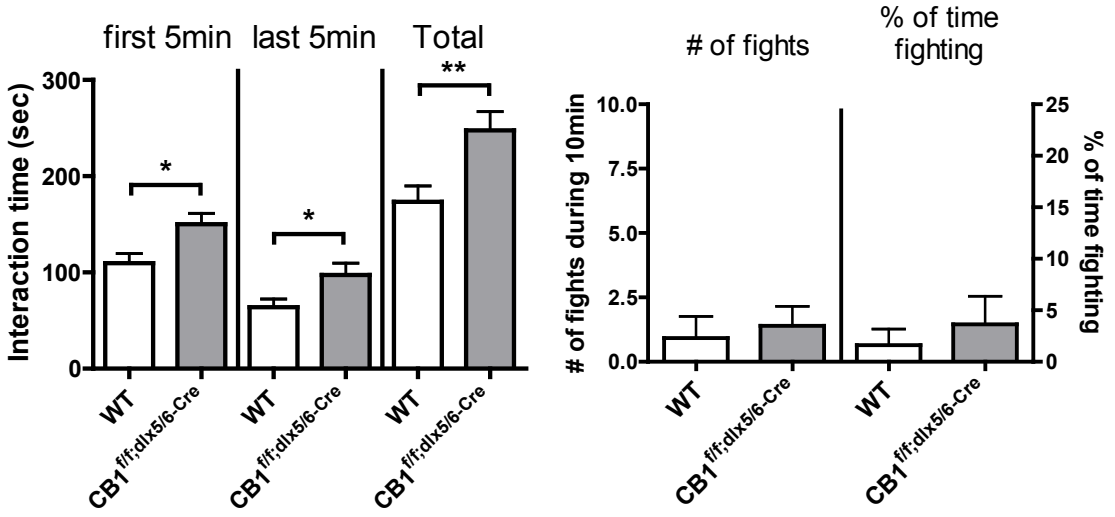

Fig. 40: Animate exploration in the resident-intruder test. Social interaction with an unknown, younger intruder (left) and number of fights induced by the resident and percentage of time fighting (right) for CB1$^{f/f;dlx5/6\text{-}Cre}$ mice compared to respective WT littermates. CB1$^{f/f;dlx5/6\text{-}Cre}$ displayed an increased interaction with the intruder compared to their wild-type control littermates, but no difference in aggressive behaviour, *p<0.05, **p<0.01(t test; interaction time n=15/WT and n=14/KO; fights n=13/WT and n=15/KO; fighting time n=15/WT and n=16/KO; male animals, 5-6.5 months old).

CB1$^{f/f;NEX\text{-}Cre}$ mice exhibited a significantly decreased interaction with the intruder animals for the 10 min interaction phase as compared to wild-type animals (T_{35}=2.297, p=0.0277; Fig. 41 left). By splitting the 10 min period into two 5 min bins it became obvious that the difference in interaction was mainly visible for the first 5 min bin (T_{35}=3.106, p=0.0038). Additionally, CB1$^{f/f;NEX\text{-}Cre}$ animals displayed an altered aggressive behaviour (Fig. 41 right). Even though the number of fights was not different between the genotypes (T_{35}=0.8932; p=0.3778), the time that CB1$^{f/f;NEX\text{-}Cre}$ mice spent fighting with the intruder was significantly increased (T_{35}=2.249; p=0.0309). Additional analysis revealed that CB1$^{f/f;NEX\text{-}Cre}$ wild-type animals display a significant increase in aggression compared to the other control groups, CB1$^{f/f;D1\text{-}Cre}$ wild-type mice and CB1$^{f/f;dlx5/6\text{-}Cre}$ wild-type animals. Thus, differences were detected in the number of fights (Kruskal-Wallis statistic=7.478, p=0.0238; Dunn's Multiple Comparison Post-Test: CB1$^{f/f;D1\text{-}Cre}$ wild-type mice vs. CB1$^{f/f;NEX\text{-}Cre}$ controls, p>0.05; CB1$^{f/f;D1\text{-}Cre}$ wild-type mice vs. CB1$^{f/f;dlx5/6\text{-}Cre}$ controls, p>0.05; CB1$^{f/f;NEX\text{-}Cre}$ controls vs. CB1$^{f/f;dlx5/6\text{-}Cre}$ wild-type controls, p<0.05) and for the percentage of time fighting (Kruskal-Wallis statistic=7.584; p=0.0226; Dunn's Multiple Comparison Post-Test: CB1$^{f/f;D1\text{-}Cre}$ wild-type mice vs. CB1$^{f/f;NEX\text{-}Cre}$ controls, p>0.05; CB1$^{f/f;D1\text{-}Cre}$ wild-type mice vs. CB1$^{f/f;dlx5/6\text{-}Cre}$ controls, p>0.05; CB1$^{f/f;NEX\text{-}Cre}$ controls vs. CB1$^{f/f;dlx5/6\text{-}Cre}$ wild-type controls, p<0.05) as well.

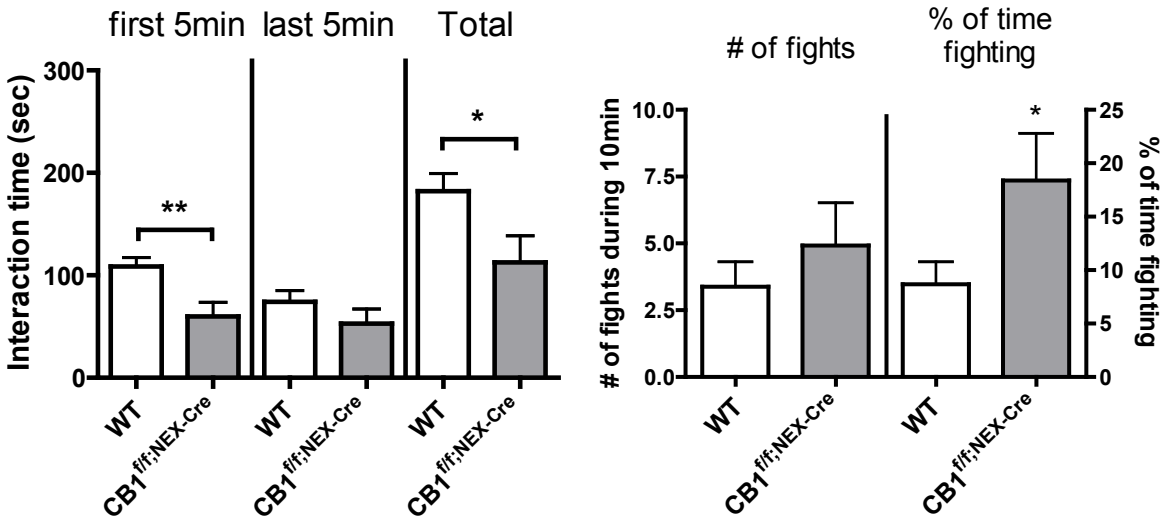

Fig. 41: Animate exploration in the resident-intruder test. Social interaction with an unknown, younger intruder (left) and number of fights induced by the resident and percentage of time fighting (right) for $CB1^{f/f;NEX\text{-}Cre}$ mice compared to wild-type littermate controls. $CB1^{f/f;NEX\text{-}Cre}$ animals showed a significantly reduced exploration during the first 5 min observation period and an increased aggression towards the intruder when compared to wild-type littermates., *p<0.05, **p<0.01(t test; n=24/WT and n=13/KO; male animals, 5-9 months old).

5.1.5 Models of addiction

Drug-induced reinforcement can be assessed directly in mice. Different ways can be used. First, there is the possibility to use either operant or non-operant self-administration methods. Second, the use of classical conditioning-based paradigms, such as conditioned place preference, conditioned place aversion or sign tracking, by facilitation of intracranial electric self-stimulation, or, alternatively by drug-induced memory enhancement (Sanchis-Segura and Spanagel, 2006). I decided to test the $CB1^{f/f;D1\text{-}Cre}$ mouse line in the classical conditioning-based paradigms and used the conditioned place preference test.

5.1.5.1 Conditioned place preference test

Before starting with the main experiments by testing the mice in conditioned place preference I first performed pilot experiments.

5.1.5.1.1 Establishing of the model

By the use of the well-established drug cocaine in this paradigm (Eisener-Dorman et al. 2011; Martin et al. 2000), I tested the model and the experimental protocol with C57BL/6N mice.

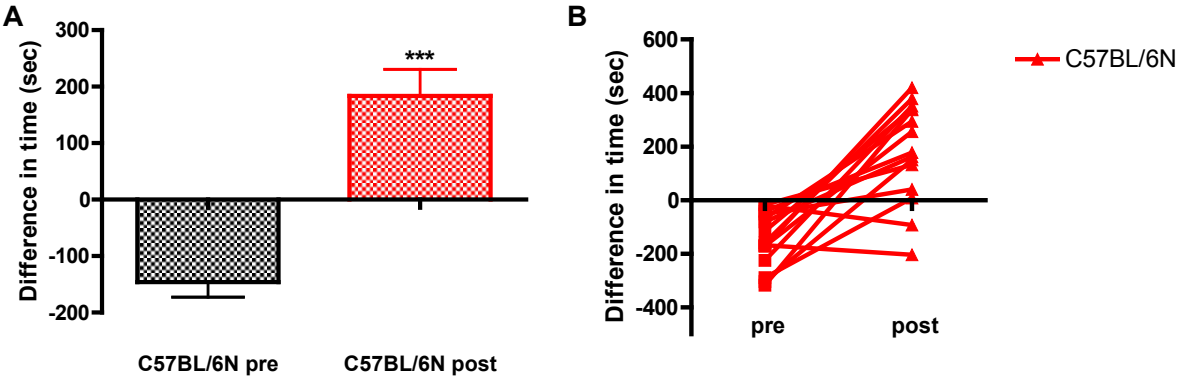

Fig. 42: Effect of cocaine (10 mg/kg) in the three compartment biased conditioned place preference model in pre and post test. (**A**) Cocaine significantly changes the natural preference of the animals towards the drug-associated compartment after four days of conditioning in wild-type C57BL/6N mice. (**B**) Difference of time spent in the drug associated vs. vehicle associated compartments shown for all mice separately. ***p<0.0001(t test; n=15; male animals, 3 months old).

Fig. 42 presents the effect of cocaine (10.0 mg/kg) in increasing the time spent in the drug-associated compartment in C57BL/6N animals. Cocaine significantly increased the time spent in the drug-associated compartment (183.5 ± 47.28 sec) in C57BL/6N mice compared to the time spent in this compartment before the conditioning (-146.1 ± 26.42 sec). Student´s t test revealed that this effect of drug was significant (T_{28}=6.087; p<0.0001).

5.1.5.1.2 Evaluating of nicotine dose

In order to find the best working dose of nicotine for our conditional mutant mouse line I accomplished a dose-response curve with C57BL/6N animals. The animals were tested for analgesia in the hotplate test and for locomotion in the open field test under treatment of vehicle and four different doses of nicotine. The highest dose, which did not cause significant analgesic or locomotor effects, was used for further CPP experiments with the $CB1^{f/f;D1\text{-}Cre}$ mouse line.

Fig. 43 displays the effect of different nicotine injections (0.1, 0.25, 0.5 and 1.0 mg/kg) and vehicle injection on locomotor activity in an open field test of five minutes. Only the group injected intraperitonally with the 1.0 mg/kg dose of nicotine showed a significant decrease

(T_{14}=4.577; p=0.0004) in the distanced moved (1138 ± 156.1 cm) compared to vehicle control group (2038 ± 119.6 cm).

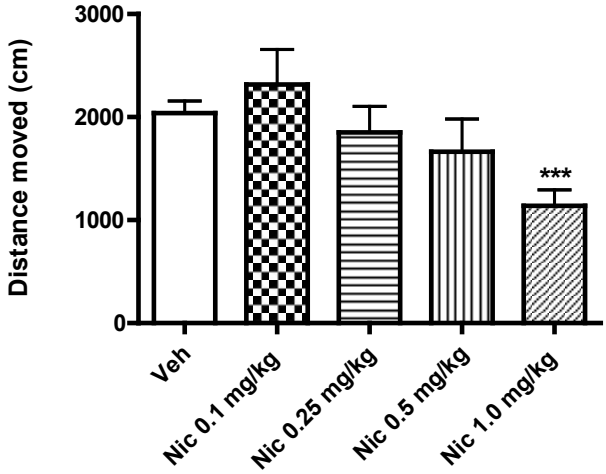

Fig. 43: Effect of different doses of nicotine on locomotion in C57BL/6N mice. Nicotine (1.0 mg/kg) significantly decreased the distance moved compared to vehicle injected mice. ***p< 0.001 (t test; n=8/group; male animals, 3-4 months old).

Additionally, the effect of different nicotine injections (0.1, 0.25, 0.5 and 1.0 mg/kg) and vehicle injection on analgesia in the hotplate test is shown in Fig. 44. Here as well only the group injected intraperitonally with the 1.0 mg/kg dose of nicotine exhibited a significant increase (T_{14}=3.764; p=0.0021) in the escape latency (13.03 ± 0.7671 sec) compared to the vehicle control group (8.725 ± 0.8464 sec).

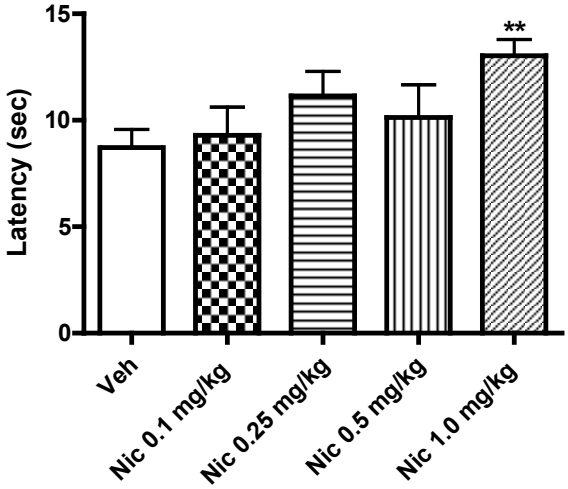

Fig. 44: Effect of different doses of nicotine on hot plate escape latency in C57BL/6N mice. Nicotine (1.0 mg/kg) significantly increased the reaction time compared to vehicle injected mice. **p< 0.01 (t test; n=8/group; male animals, 3-4 months old).

5.1.5.1.3 Performance of conditioned place preference test

Next, I tested the conditioned place preference by the use of nicotine (0.5 mg/kg) in $CB1^{f/f;D1\text{-}Cre}$ animals as compared to their wild-type littermates. The effect of nicotine (0.5 mg/kg) increased the time spent in the drug-associated compartment in wild-type and $CB1^{f/f;D1\text{-}Cre}$ animal, as shown in Fig. 45. In this CPP, the effect of drug was significant ($F_{1,34}$=8.62; p=0.0059) in the wild-type animals of $CB1^{f/f;D1\text{-}Cre}$ mice. However, no genotype-related differences could be seen ($F_{1,34}$=0.75; p=0.3922). In other words, nicotine increased the time spent in the drug-associated compartment (-83.89 ± 56.71 sec) in $CB1^{f/f;D1\text{-}Cre}$ mice compared to the time spent in this compartment before the conditioning (-192.5 ± 46.95 sec) and in the wild-type animals (33.75 ± 43.38 sec) as compared to the time spent in this compartment before the conditioning (-128.4 ± 43.12 sec). But this effect was only significant for the wild-type littermates (T_{14}=2.652; p=0.019).

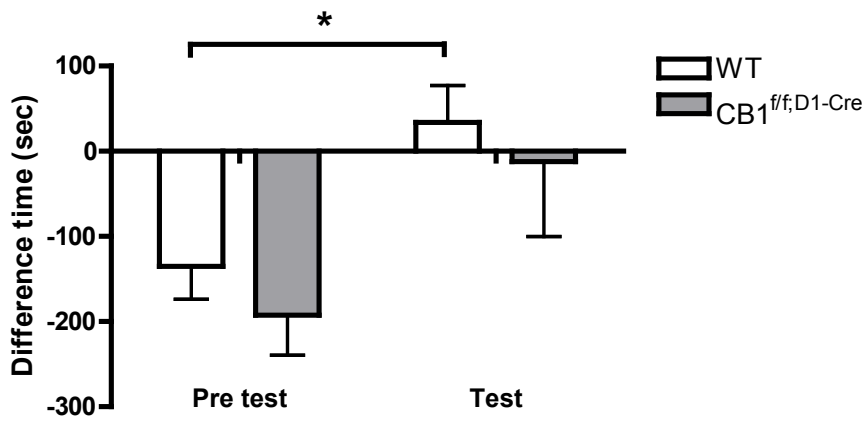

Fig. 45: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in $CB1^{f/f;D1\text{-}Cre}$. Nicotine significantly increased the time spent in the drug-associated compartment in WT mice, while there was no significant effect in $CB1^{f/f;D1\text{-}Cre}$ animals. *p< 0.05 (2-WAY ANOVA followed by Bonferroni test; n=10/group, male animals, 4-6 months old).

A repetition of the first CPP with another batch of $CB1^{f/f;D1\text{-}Cre}$ mice revealed no significance at all (Fig. 46). Both genotypes showed no significant change in time spent in the drug-associated compartment and no significant interaction between the genotype and drug factor could be observed ($F_{1,33}$=1.04; p=0.3163). Additionally, no further significance could be found, neither in the effect of genotype ($F_{1,33}$=1.07; p=0.3082) nor in the effect of injection ($F_{1,33}$=1.31; p=0.2611).

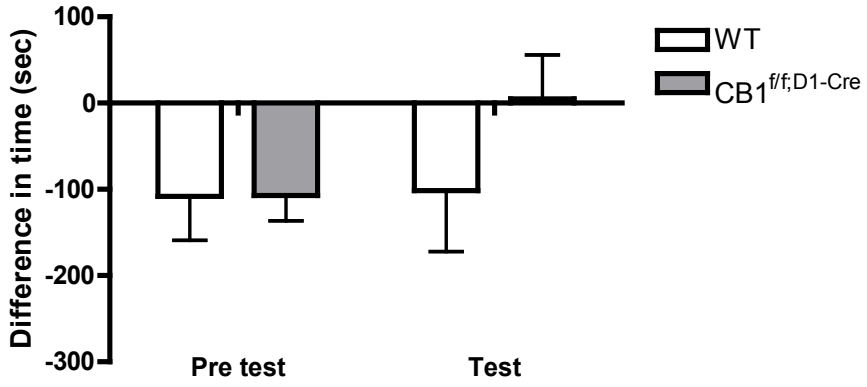

Fig. 46: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in CB1$^{f/f;D1\text{-}Cre}$ mice. Nicotine increased the time spent in the drug-associated compartment in CB1$^{f/f;D1\text{-}Cre}$ animals while no effect was seen in respective wild-type littermates. (2-WAY ANOVA followed by Bonferroni test; n=10/group, male animals, 4-6 months old).

In order to compare the data obtained from CB1$^{f/f;D1\text{-}Cre}$ mice, I performed an additional CPP with the CB1 null-mutant animals CB1$^{-/-}$, as shown in Fig. 47.

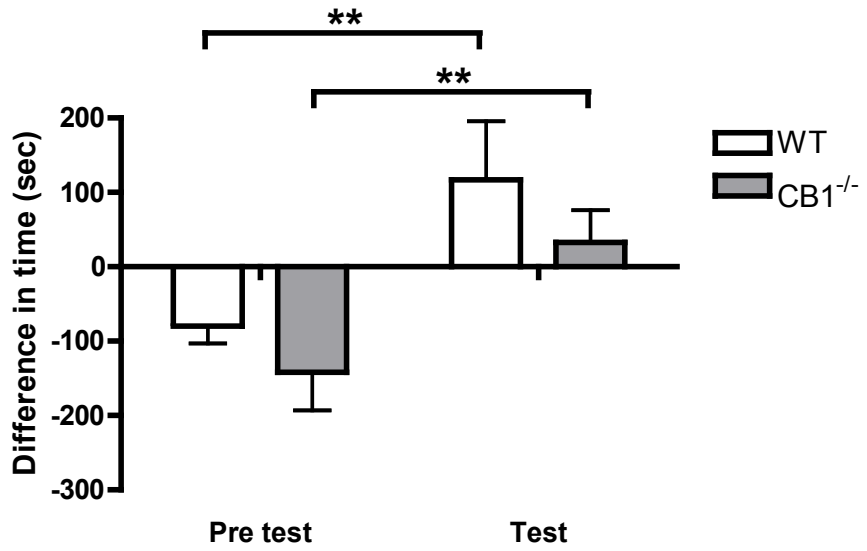

Fig. 47: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in CB1$^{-/-}$ animals. Nicotine significantly increased the time spent in the drug-associated compartment both in CB1$^{-/-}$ mice and their wild-type littermates. **p< 0.01 (2-WAY ANOVA followed by Bonferroni test; n=9/group, male animals, 5-6 months old).

Nicotine (0.5 mg/kg) significantly increased the time spent in the drug-associated compartment in wild-type and CB1$^{-/-}$ animals. With this dose of the drug, a significant effect of condi-

tioning ($F_{1,32}$=12.35; p=0.0013) was observed in both groups, but this effect was independent of the genotype ($F_{1,32}$=1.92; p=0.1760). Nicotine increased the time spent in the drug-associated compartment both in CB1$^{-/-}$ mice and in wild-type animals. CB1$^{-/-}$ spent significantly more time in the drug-associated compartment (51.21 ± 33.87 sec) compared to the time spent in this compartment before the conditioning (-152.4 ± 47.05 sec). This could be seen as well in the wild-type animals (127.4 ± 76.75 sec) compared to the time spent in this compartment before the conditioning (-98.06 ± 25.33 sec).

No differences could be found in comparison of the time spent in drug-associated compartment of the mice of CB1$^{-/-}$ and CB1$^{f/f;D1\text{-}Cre}$ line. Wild-type animals of CB1$^{-/-}$ spent in a naïve state -98.06 ± 25.33 sec in the drug-associated compartment while wild-type animals of CB1$^{f/f;D1\text{-}Cre}$ spent in the first CPP -135.3 ± 38.68 sec and in the second one -84.08 ± 31.00 sec. The comparison of the mutants of both lines exhibited the same effect. CB1$^{-/-}$ mice spent the same amount of time (-152.4 ± 47.05 sec) in the drug-associated compartment as the mutants of CB1$^{f/f;D1\text{-}Cre}$ line (-192.5 ± 46.95 sec for the first and -84.08 ± 31.00 sec for the second CPP). Student's t test did not reveal any significant differences. A closer look to the results of both CPP with CB1$^{f/f;D1\text{-}Cre}$ line showed the individual time change for each single mouse tested (Fig. 48).

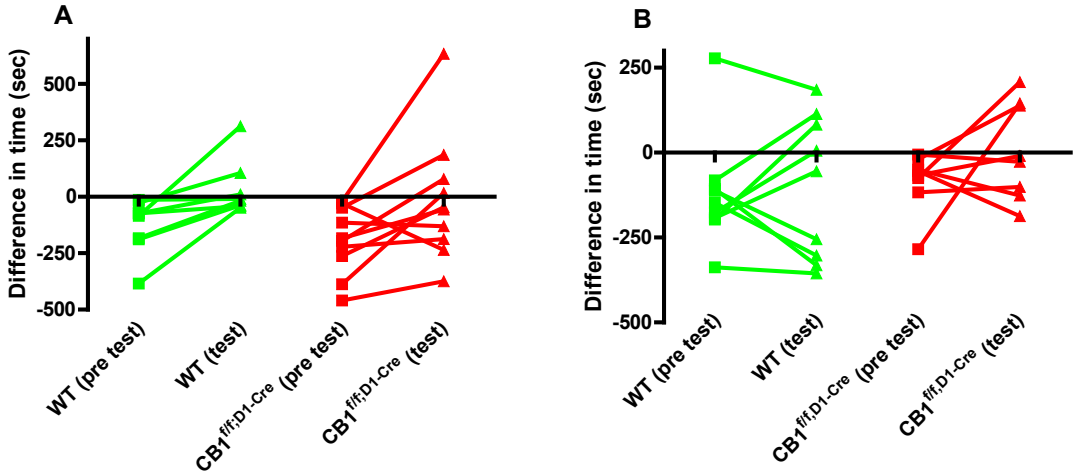

Fig. 48: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in CB1$^{f/f;D1\text{-}Cre}$ mice. The behaviour of each mouse is presented individually for the first (A) and the second (B) CPP made.

By presenting the data of both CPP in this way, it can be seen that the effect of increasing time in drug-associated compartment was not homogenous for all animals. In fact, the complete lack of place preference in the second experiment is most probably due to the extremely high variability of the reference (control) group. This led to the decision to test naïve ani-

mals of the C57BL/6N background and to compare those animals with naïve mice of C57BL/6J background in order to gain insight to possible background differences.

5.1.5.1.4 Evaluating of background differences in conditioned place preference test

Next, I tested the conditioned place preference by the use of nicotine (0.5 mg/kg) in wild-type animals of C57BL/6N and C57BL/6J background. Fig. 49 presents the effects of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment of both wild-type strains. In this CPP, nicotine significantly (T_{18}=3.124; p=0.0059) increased the time spent in the drug-associated compartment (30.11 ± 37.20 sec) in C57BL/6J mice compared to the time spent in this compartment before the conditioning (-121.5 ± 31.16 sec), but the increase of time spent in the drug-associated compartment (12.56 ± 66.97 sec) compared to the time spent in this compartment before the conditioning (-122.2 ± 25.65 sec) was not significant in C57BL/6N mice (T_{18}=1.879; p=0.0765).

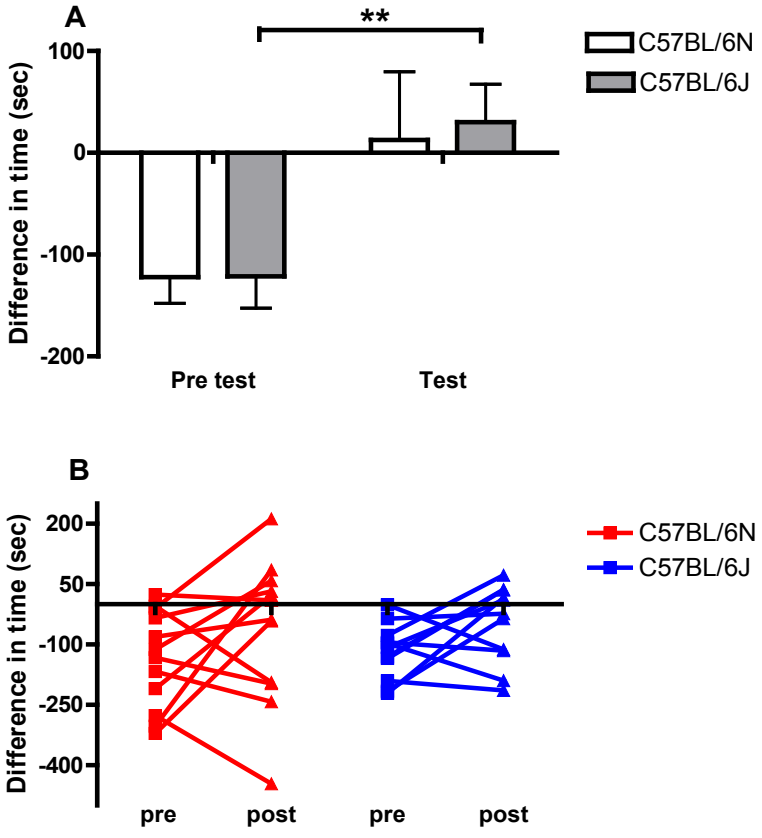

Fig. 49: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in C57BL/6N and C57BL/6J animals. (**A**) Nicotine effect in both groups. (**B**) Nicotine effect presented for each mouse individually. Nicotine significantly increased the time spent in the drug-associated compartment in C57BL/6J mice, but not in C57BL/6N mice. ** p<0.01 (t test; n=10/group, male animals, 11-12 weeks old).

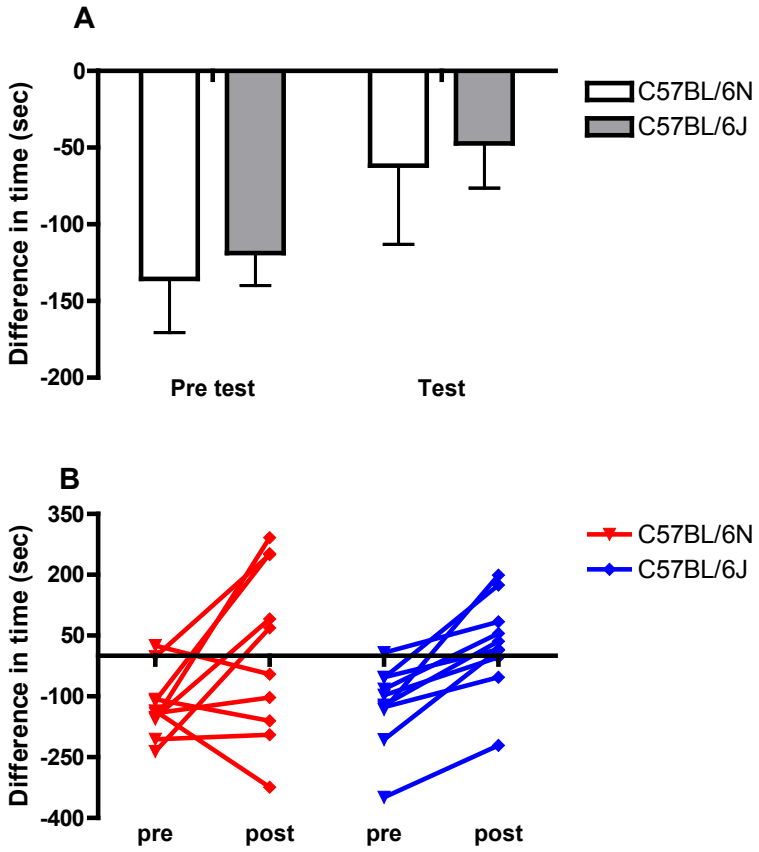

Fig. 50: Effect of nicotine (0.5 mg/kg) increasing the time spent in the drug-associated compartment in C57BL/6N and C57BL/6J animals. (**A**) Nicotine effect in the both groups. (**B**) Nicotine effect presented for each mouse individually. Nicotine increased the time spent in the drug-associated compartment in both groups, but this effect was not significant (t test; n=12/ C57BL/6N and n=11/ C57BL/6J, male animals, 10-12 weeks old).

An additional repetition of this CPP was made. The results are shown in Fig. 50. This time, nicotine (0.5 mg/kg) increased the time spent in the drug-associated compartment of both wild-type strains, but the effect was not significant. During the test C57BL/6N mice spent more time in drug-associated compartment (-61.72 ± 51.43 sec) compared to the time spent in this compartment before the conditioning (-135.6 ± 34.91 sec), but this increase of time was not significant (T_{22}=1.189; p=0.2473). C57BL/6J animals spent as well more time in the drug-associated compartment after conditioning (-47.32 ± 29.12 sec) than before conditioning (-118.8 ± 21.08 sec). This effect was not significant either (T_{20}=1.989; p=0.0606), but showed a tendency towards significance.

By crossing both lines once into a F1 hybrid C57BL/6N x C57BL/6J (named C57BL/6NJ) strain, I tried to evaluate the effects of strains. With the hybrids another CPP has been performed and the results can be seen in Fig. 51. The C57BL/6NJ hybrids showed a similar behaviour as the C57BL/6N strain. Nicotine increased the time spent in drug-associated com-

partment (-44.24 ± 30.02 sec) compared to the time spent in this compartment before the conditioning (-89.18 ± 10.88 sec), but this increase of time was not significant (T_{56}=1.407; p=0.1648).

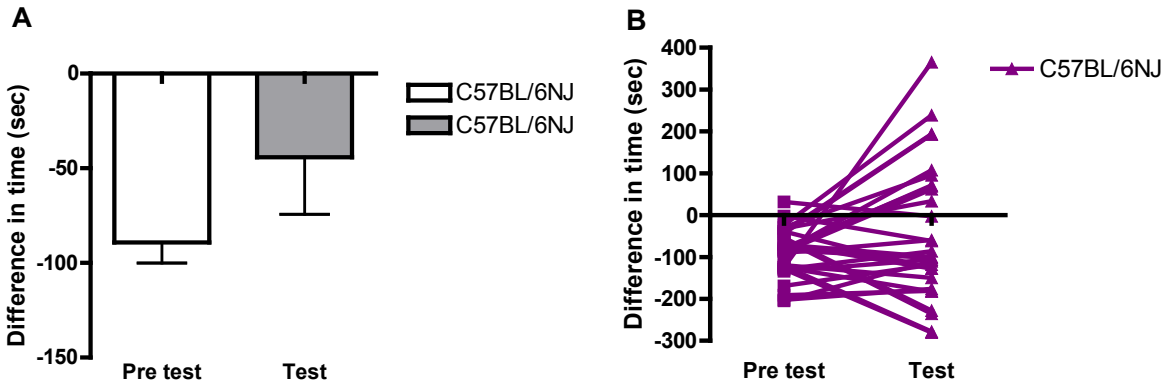

Fig. 51: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in C57BL/6J animals. (**A**) Nicotine effect in the whole group. (**B**) Nicotine effect presented for each mouse individually. Nicotine increased the time spent in the drug-associated compartment in both groups, but this effect was not significant (t test; n=29, male animals, 10-12 weeks old).

5.1.5.1.5 Conditioned place preference test for gene array analysis

Another conditioned place preference (CPP) experiment with nicotine injections was performed in order to gain further insights to processes and gene alterations in the brain of $CB1^{f/f;D1\text{-}Cre}$ mice after CPP.

Based on their distinct behaviour, the decision was made to separate the animals into certain groups for the additional validation with a GeneChip Mouse Genome 430A 2.0 Array from Affymetrix. Fig. 52 represents the effect of repeated nicotine (0.5 mg/kg) injections in $CB1^{f/f;D1\text{-}Cre}$ animals.

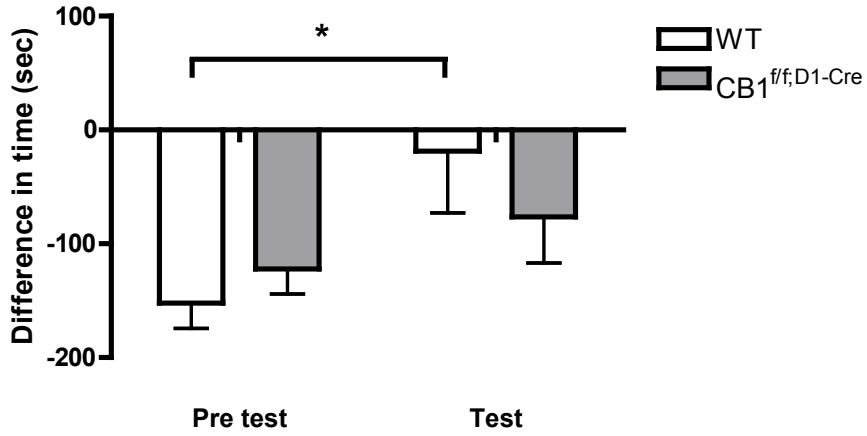

Fig. 52: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in CB1$^{f/f;D1\text{-}Cre}$. Nicotine significantly increased the time spent in the drug-associated compartment in wild-type controls, but no effect was found in CB1$^{f/f;D1\text{-}Cre}$ animals. *p< 0.05 (2-WAY ANOVA followed by Bonferroni test; n=15/group, male animals, 6-7 months old).

As can be seen, nicotine increased the time spent in the drug-associated compartment (-76.33 ± 40.44 sec) in CB1$^{f/f;D1\text{-}Cre}$ mice compared to the time spent in this compartment before the conditioning (-122.1 ± 22.00 sec) and in the wild-type animals (-18.61 ± 54.16 sec) compared to the time spent in this compartment before the conditioning (-152.2 ± 22.08 sec). But this effect was only significant for the wild-type littermates (T_{28}=2.284; p= 0.0302) and not for the CB1$^{f/f;D1\text{-}Cre}$ animals (T_{28}=0.9932; p= 0.3921). Analysis did not reveal a significant genotype effect ($F_{1,56}$=0.14; p=0.7123). But drug effect was significant ($F_{1,56}$=5.80; p=0.0193) in the wild-type animals of CB1$^{f/f;D1\text{-}Cre}$ mice.

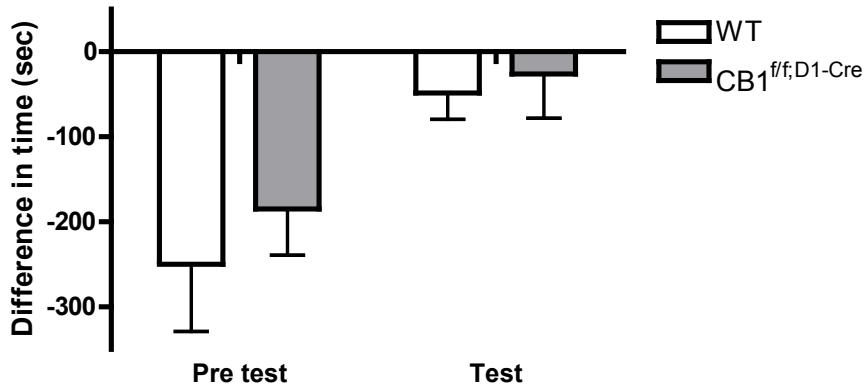

Fig. 53: Effect of vehicle injections on the time spent in the drug-associated compartment in CB1$^{f/f;D1\text{-}Cre}$. Saline increased the time spent in the drug-associated compartment in wild-type controls and in CB1$^{f/f;D1\text{-}Cre}$ controls. *p<0.05 (2-WAY ANOVA followed by Bonferroni test; n=4/group, male animals, 6-7 months old).

Additionally, the control animals, which received only vehicle during the conditioning period, reduced their time spent in drug-associated compartment (Fig. 53). For control groups no significant interaction was reached between genotype and conditioning factors ($F_{1,9}$=0.11; p=0.7434). A significant effect of conditioning ($F_{1,9}$=8.17; p=0.0188) was observed in both groups, but this effect was independent of the genotype ($F_{1,9}$=0.48; p=0.5072).

5.1.5.1.6 Conditioned place preference test with backcrossed J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals and J-CB1$^{-/-}$ mice

After all different conditioned place preference (CPP) experiments with the mutant line on C57BL/6N background, which produced data with particularly high variability, I backcrossed the CB1$^{-/-}$ and CB1$^{f/f;D1\text{-}Cre}$ mice for more than 10 generations into C57BL/6J background and received the so called J-CB1$^{-/-}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line, respectively.

5.1.5.1.6.1 Conditioned place preference test for the effect of nicotine injections in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice

First, I repeated a CPP with nicotine injections (0.5 mg/kg) under the same conditions as before for the gene array. The results of this CPP with the first batch of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice are presented in Fig. 54. Both genotypes showed significant changes in time spent in drug-associated compartment. Nicotine increased the time spent in the drug-associated compartment (14.39 ± 47.51 sec) in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice compared to the time spent in this compartment before the conditioning (-129.4 ± 31.09 sec) and in the wild-type animals (6.700 ± 42.16 sec) compared to the time spent in this compartment before the conditioning (-158.5 ± 28.72 sec). This effect was significant for both J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals (T_{14}=2.532; p=0.0239) and their respective wild-type littermates (T_{14}=3.239; p=0.0059). However, no genotype-related differences could be found in the effect of nicotine on place preference ($F_{1,28}$=0.23; p=0.6332).

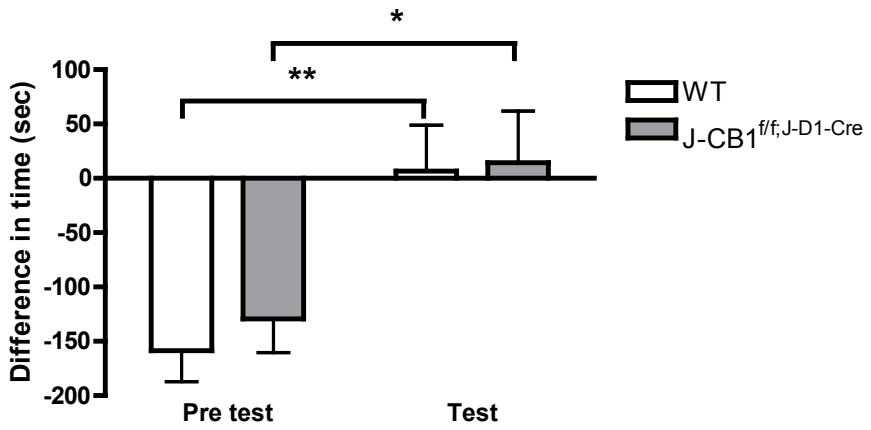

Fig. 54: Effect of nicotine (0.5 mg/kg) on the time spent in the drug-associated compartment in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$. Nicotine significantly increased the time spent in the drug-associated compartment in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals and in their respective wild-type littermates. *p<0.05 and **p<0.01 (2-WAY ANOVA followed by Bonferroni test; n=8/group, male animals, 3-5 months old).

The control groups of both genotypes, which received only vehicle during the conditioning period, increased their time spent in drug-associated compartment as well (Fig. 55), but this effect was not significant. Both genotypes showed no significant interaction between genotype and conditioning factors ($F_{1,20}$=0.09; p=0.7726). A significant effect of conditioning ($F_{1,20}$=6.58; p=0.0184) was observed in both groups, but this effect was independent of the genotype ($F_{1,20}$=0.12; p=0.7336).

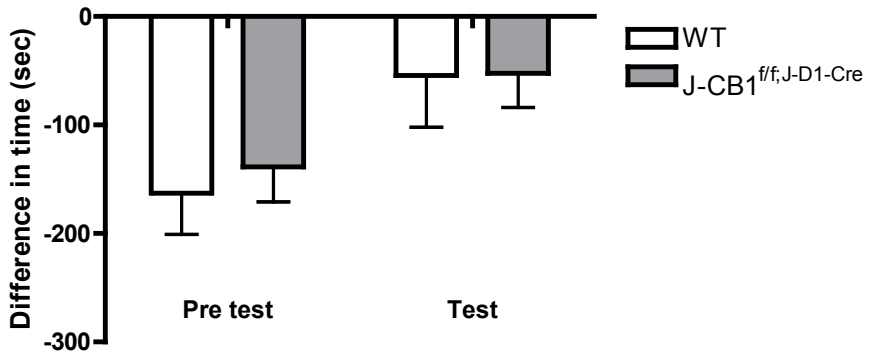

Fig. 55: Effect of vehicle injections on the time spent in the drug-associated compartment in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$. Saline increased the time spent in the drug-associated compartment in wild-type controls and in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ controls. (2-WAY ANOVA followed by Bonferroni test; n=6/group, male animals, 3-5 months old).

5.1.5.1.6.2 Conditioned place preference test for the effect of cocaine injections in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ and in J-CB1$^{-/-}$ mice

As I could show that backcrossing of the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ line was successful in producing more homogenous data for drug conditioning with nicotine (0.5 mg/kg), I decided to evaluate the effect of cocaine injections as well in this model of addiction.

First I performed a CPP experiment with a moderate dose of cocaine (10 mg/kg) in both J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ and in J-CB1$^{-/-}$ animals. Fig. 56 presents the results of the effect of cocaine (10 mg/kg) in increasing the time spent in the drug-associated compartment in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice.

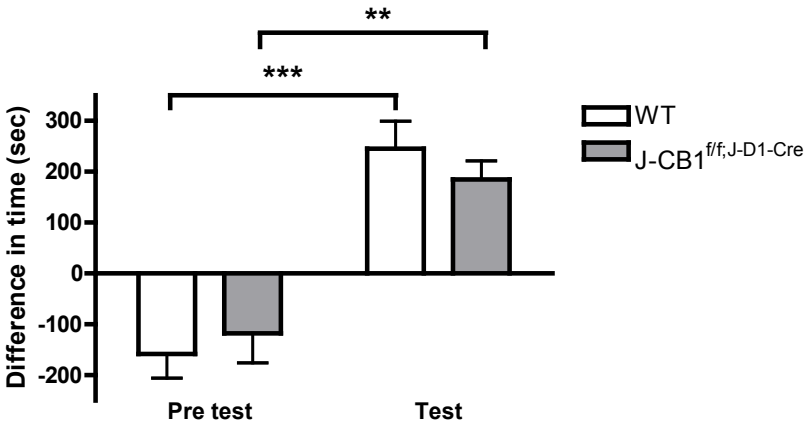

Fig. 56: Effect of cocaine (10 mg/kg) on the time spent in the drug-associated compartment in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$. Cocaine significantly increased the time spent in the drug-associated compartment in both J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice and WT littermates. **p<0.01 and ***p<0.001 (2-WAY ANOVA followed by Bonferroni test; n=6/group, male animals, 3-4.5 months old).

As presented, cocaine significantly increased the time spent in the drug-associated compartment (184.8 ± 36.32 sec) in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice compared to the time spent in this compartment before the conditioning (-117.9 ± 57.70 sec) and in their respective wild-type littermates (245.3 ± 54.19 sec) compared to the time spent in this compartment before the conditioning (-158.1 ± 47.85 sec). But this effect was stronger for the wild-type littermates (T_{10}=5.579; p=0.0002) than for the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals (T_{10}=4.440; p=0.0013). 2-WAY ANOVA analysis did not reveal a significant interaction for genotype and the drug factor ($F_{1,20}$=1.39; p=0.3234) or for the genotype effect ($F_{1,20}$=0.04; p=0.8408). Nevertheless, the drug effect was significant ($F_{1,20}$=50.49; p<0.0001) in both genotypes.

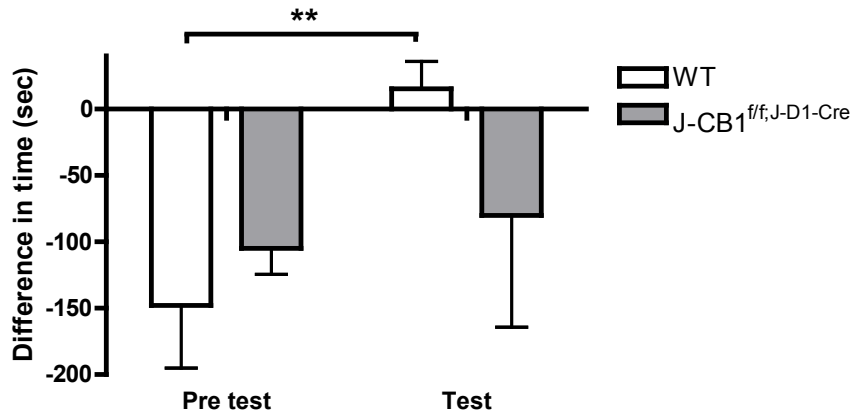

Fig. 57: Effect of vehicle injections on the time spent in the drug-associated compartment in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$. Saline increased the time spent in the drug-associated compartment in wild-type controls and in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ controls, but this effect was only significant in wild-type littermates. **p<0.01 (2-WAY ANOVA followed by Bonferroni test; n=6/group, male animals, 3-4.5 months old).

The control groups of both genotypes, which received only saline injections for the time of conditioning, spent more time in drug-associated compartment as well (Fig. 57), but this effect was only significant for the wild-type animals found by student´s t test (T_{10}=3.173; p=0.0099). Neither a significant effect of conditioning ($F_{1,20}$=3.49; p=0.0764) nor an effect of the genotype ($F_{1,20}$=0.27; p=0.6082) was observed in both groups.

Identically to this experiment, I used animals of the CB1 null-mutant mouse line (J-CB1$^{-/-}$) in the same paradigm in order to gain results of the behaviour of the null-mutant mouse line as well. Fig. 58 presents the results of the J-CB1$^{-/-}$ mice. Cocaine (10 mg/kg) significantly increased the time spent in the drug-associated compartment in wild-type and J-CB1$^{-/-}$ animals. With this dose of the drug, no significant interaction was reached between genotype and conditioning factors ($F_{1,26}$=0.74; p=0.3965). However, a very significant effect of injections during conditioning ($F_{1,26}$=77.29; p<0.0001) was observed in both groups, but this effect was independent of the genotype ($F_{1,26}$=0.50; p=0.4856). Cocaine increased the time spent in the drug-associated compartment in J-CB1$^{-/-}$ mice as in wild-type animals. J-CB1$^{-/-}$ spent significantly more time in the drug-associated compartment (269.1 ± 47.27 sec) compared to the time spent in this compartment before the conditioning (-134.7 ± 28.05 sec). This could be seen as well in the wild-type animals (203.4 ± 56.01 sec) compared to the time spent in this compartment before the conditioning (-128.2 ± 30.33 sec).

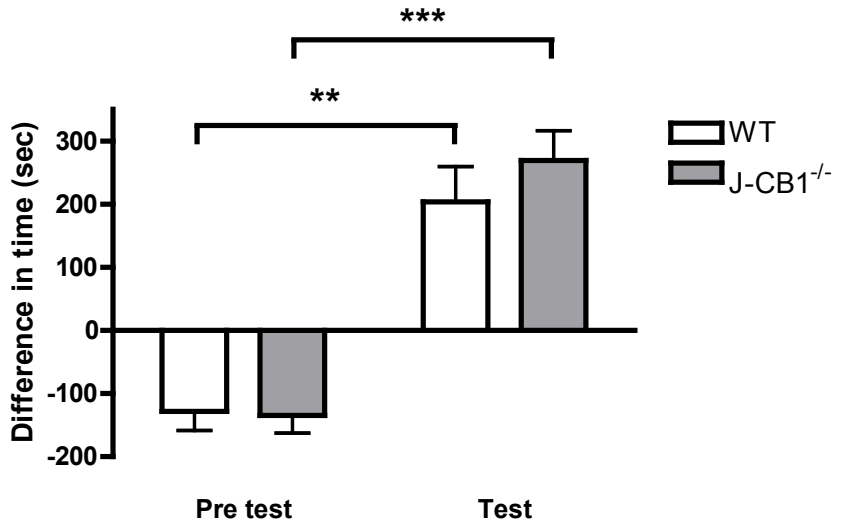

Fig. 58: Effect of cocaine (10 mg/kg) on the time spent in the drug-associated compartment in J-CB1$^{-/-}$ animals. Cocaine significantly increased the time spent in the drug-associated compartment in both J-CB1$^{-/-}$ mice and their respective WT littermates. **p<0.01 and ***p<0.001 (2-WAY ANOVA followed by Bonferroni test; n=8/J-CB1$^{-/-}$ and n=7/WT, male animals, 2.5-4.5 months old).

With the help of the more sophisticated program SPSS statistics 19.0 I was able to show in Tab. 5 that there is actually an effect or interaction between genotype and treatment in J-CB1$^{-/-}$ knock-out animals.

origin	Sum of squares of type III	df	means of squares	F	sig.
corrected model	861159.219[a]	5	172231.844	5.422	0.002
constant term	5099.043	1	5099.043	0.161	0.693
genotype	104365.464	1	104365.464	3.285	0.084
treatment	743398.159	1	743398.159	23.402	0.000***
pre test	4070.210	1	4070.210	0.128	0.724
genotype * treatment	142249.520	1	142249.520	4.478	0.046*
genotype * pre test	99732.430	1	99732.430	3.140	0.091
error	667084.821	21	31765.944		
overall	1764284.790	27			
corrected overall variation	1528244.040	26			

Table 5: Genotype effects dependent of treatment and time. Interaction of genotype and treatment and treatment alone showed a significant effect in J-CB1$^{-/-}$ animals and their wild-type littermates. *p<0.05 and p<0.0001, a=R-square =0.563 (corrected R-square =0.460)

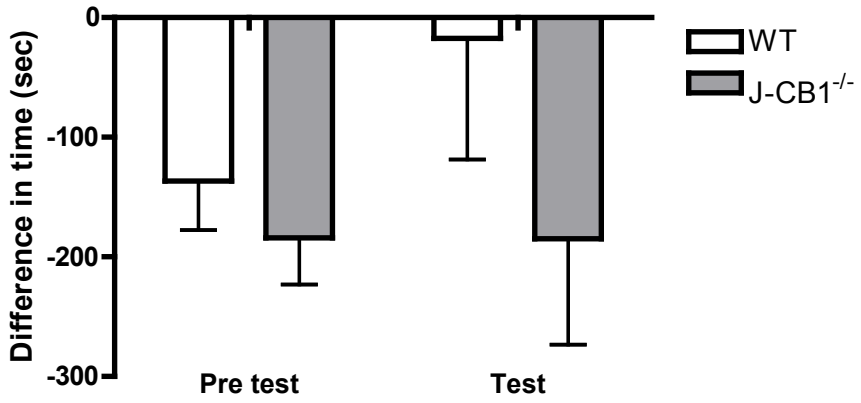

Fig. 59: Effect of vehicle injections on the time spent in the drug-associated compartment in J-CB1$^{-/-}$ mice. Saline increased the time spent in the drug-associated compartment in wild-type controls and not in J-CB1$^{-/-}$ controls, but this effect was not significant (2-WAY ANOVA followed by Bonferroni test; n=6/group, male animals, 2.5-4.5 months old).

Both control groups of each genotype received the entire time vehicle during the conditioning period. These injections increased the time spent in drug-associated compartment only in wild-type littermates of J-CB1$^{-/-}$ mice (Fig. 59), but this effect was not significant. Both genotypes showed no significant interaction between genotype and conditioning factors ($F_{1,18}$=0.65; p=0.4311). No significant changes in time spent in the drug-associated compartment could be seen neither in the effect of conditioning ($F_{1,18}$=0.63; p=0.4368) or in the effect of genotype ($F_{1,18}$=2.08; p=0.1662).

No differences could be found in comparison of the time spent in the drug-associated compartment of the mice of J-CB1$^{-/-}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ line in the pre test. Wild-type animals of J-CB1$^{-/-}$ spent in a naïve state -128.2 ± 30.33 sec while wild-type animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ spent -158.1 ± 47.85 sec in the drug-associated compartment (T_{11}=0.5438; p=0.5975). The comparison of the knock-outs of both lines exhibited the same effect before conditioning.

J-CB1$^{-/-}$ mice spent the same amount of time (-134.7 ± 28.05 sec) in the drug-associated compartment as the mutants of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ line (-117.9 ± 57.70 sec). Student´s t test did not reveal any significant differences for the pre test (T_{10}=0.2839; p=0.7813). The same effect could be detected for the time spent in the drug-associated compartment of the mice of J-CB1$^{-/-}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ line in the test after conditioning. The knock-out animals of J-CB1$^{-/-}$ line spent 269.1 ± 47.27 sec in the drug-associated compartment, while J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ knock-out mice spent 184.8 ± 36.32 sec (T_9=2.082; p=0.0709). Additionally, no significant differences were found for the wild-type littermates of both mouse lines. The wild-type ani-

mals of J-CB1-/- line spent 203.4 ± 56.01 sec in the drug-associated compartment, while J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ wild-type mice spent 245.3 ± 54.19 sec (T_{10}=0.8464; p=0.4193).

As I found already a drug effect with the moderate dose of 10 mg/kg cocaine in the J-CB1$^{-/-}$ animals, I decided to repeat the CPP experiment with another batch of J-CB1$^{-/-}$ mice and the higher dose of 20 mg/kg cocaine. Identically to the first experiment with cocaine, the 20 mg/kg dose significantly increased the time spent in the drug-associated compartment in wild-type and J-CB1$^{-/-}$ animals (Fig. 60). Similar to the lower dose of cocaine (10 mg/kg), the dose used in this CPP revealed no significant interaction between genotype and conditioning factors ($F_{1,28}$=1.85; p=0.1852). However, a very significant effect of the drug during conditioning ($F_{1,28}$=95.67; p<0.0001) was found for the groups as well, but this effect was independent of the genotype ($F_{1,28}$=2.56; p=0.1210). Cocaine increased the time spent in the drug-associated compartment independent of the genotype in both groups. J-CB1$^{-/-}$ spent significantly more time in the drug-associated compartment (290.2 ± 36.51 sec) compared to the time spent in this compartment before the conditioning (-175.8 ± 25.71 sec) (T_{14}=10.44; p<0.0001). This could be seen as well in the wild-type animals (166.5 ± 62.67 sec) compared to the time spent in this compartment before the conditioning (-185.9 ± 32.83 sec) (T_{14}=4.980; p=0.0002).

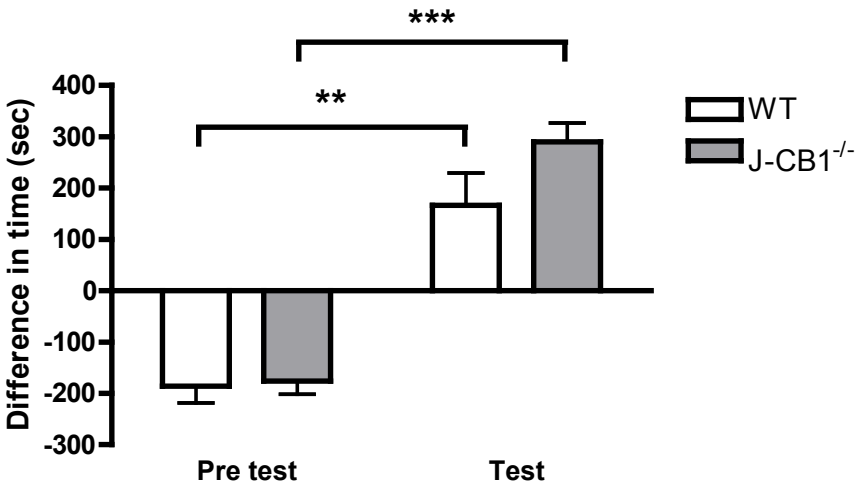

Fig. 60: Effect of cocaine (20 mg/kg) increasing the time spent in the drug-associated compartment in J-CB1$^{-/-}$ animals. Cocaine significantly increased the time spent in the drug-associated compartment in both J-CB1$^{-/-}$ mice and their respective WT littermates. **p<0.01 and ***p<0.001 (2-WAY ANOVA followed by Bonferroni test; n=8/group, male animals, 2.5-4 months old).

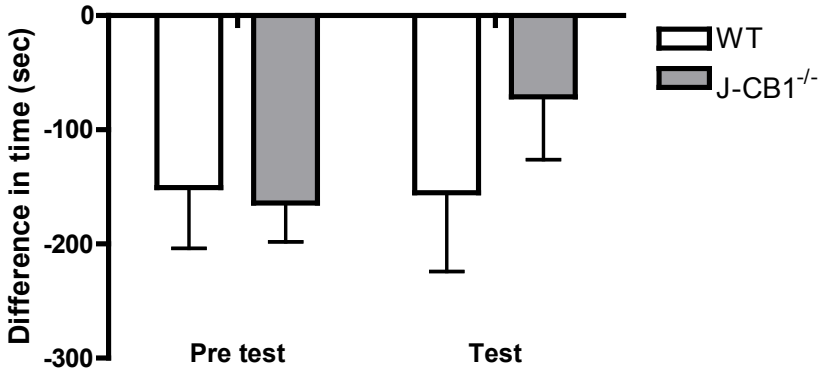

Fig. 61: Effect of vehicle injections in increasing the time spent in the drug-associated compartment in J-CB1$^{-/-}$ mice. Saline increased the time spent in the drug-associated compartment in J-CB1$^{-/-}$ controls and not in wild-type controls, but this effect was not significant (2-WAY ANOVA followed by Bonferroni test; n=6/group, male animals, 2.5-4.5 months old).

The control animals of each genotype, which were always injected with vehicle in each conditioning round, also displayed an increase in the time spent in the drug-associated compartment. Here, no significant increase could be found in one of the groups (Fig. 61). Both genotypes did not show a significant interaction between genotype and conditioning factors ($F_{1,20}$=0.81; p=0.3784) and no significant change was seen in the effect of injection ($F_{1,20}$=0.67; p=0.4230) and in the effect of genotype ($F_{1,20}$=0.42; p=0.5224).

Additionally, I compared the results of both CPP performed with cocaine with each other. By comparison of the time spent in the drug-associated compartment of the mice of J-CB1$^{-/-}$ null-mutant line in the pre test, no differences in time spent in the drug-associated compartment were revealed. Wild-type animals of J-CB1$^{-/-}$ (10 mg/kg cocaine) spent in a naïve state -203.4 ± 56.01 sec in the drug-associated compartment compared while wild-type animals of J-CB1$^{-/-}$ (20 mg/kg cocaine) spent -166.5 ± 62.67 sec (T_{13}=0.4346; p=0.6710). The mutants of both lines showed the same behaviour as before conditioning. In naïve state, J-CB1$^{-/-}$ mice (10 mg/kg cocaine) spent the same amount of time (-134.7 ± 28.05 sec) in the drug-associated compartment as the mutants of J-CB1$^{-/-}$ mice (20 mg/kg cocaine) (-175.8 ± 25.71 sec). Student´s t test did not reveal any significant differences for the pre test (T_{14}=1.081; p=0.2980). Same results were found for the time spent in the drug-associated compartment of the mice of J-CB1$^{-/-}$ in the test after conditioning with both doses of cocaine. The knock-out animals of J-CB1$^{-/-}$ line (10 mg/kg cocaine) spent 269.1 ± 47.27 sec in the drug-associated compartment, while J-CB1$^{-/-}$ knock-out mice (20 mg/kg cocaine) spent 290.2 ± 36.51 sec (T_{14}=0.3531; p=0.7293). Additionally, no significant differences were found for the wild-type littermates of both mouse lines. The wild-type animals of J-CB1$^{-/-}$ line (10 mg/kg cocaine)

spent 203.4 ± 56.01 sec compared to J-CB1$^{-/-}$ wild-type mice (20 mg/kg cocaine), which spent 166.5 ± 62.67 sec (T_{13}=0.4346; p=0.6710) in the drug-associated compartment.

5.1.5.1.6.2.1 Evaluation of locomotor activity in the conditioned place preference test

As cocaine is known to change the locomotor activity, I decided to evaluate differences between the mouse lines (Tab. 6) and dose differences as well (Tab. 7) both in naïve state and in already addicted state on the third day of conditioning.

The evaluation of the locomotor activity in the naïve state of CPP experiment revealed that only J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ knock-out mice developed a decrease in the distance moved (T_{22}=2.287, p=0.0322; Tab. 6) in the black compartment as compared to their respective wild-types. But this effect was compensated by comparing the distance moved in all compartments (T_{22}=1.082, p=0.2908; Tab. 6). J-CB1$^{-/-}$ animals did not display a change in the distance moved as compared with their respective wild-type littermates neither in the first (T_{25}=0.06468, p=0.9489, Tab. 6) nor in the second CPP experiment (T_{26}=1.513, p=0.1424); Tab. 7).

	J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ (10 mg/kg cocaine)				**J-CB1$^{-/-}$ (10 mg/kg cocaine)**			
CPP	**+/+**		**-/-**		**+/+**		**-/-**	
group	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**
pre test	**Distance moved (cm)**							
all compartments	8078 ± 235.6		7700 ± 256.8		8065 ± 287.8		8055 ± 380.1	
black compartment	3404 ± 102.3		3080 ± 97.96 *		3360 ± 125.4		3455 ± 150.2	
white compartment	2699 ± 146.8		2586 ± 142.1		2624 ± 142.0		2723 ± 115.7	
group	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**
3.conditioning	**Distance moved (cm)**							
drug-associated compartment	2810 ± 176.1	3557 ± 133.5$^{\#\#}$	2657 ± 236.2	3783 ± 195.2$^{\#\#,\circ}$	2954 ± 237.1	3849 ± 201.1$^{\#}$	2195 ± 278.1	4525 ± 166.8$^{*,\#\#\#}$

Table 6: Locomotion differences caused by cocaine (10 mg/kg). Evaluation of locomotion (distance moved), in naïve state and during 20 min conditioning in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line and J-CB1$^{-/-}$ null-mutant mouse line; +/+ (wild-type), -/- (knock-out); t-test analysis: *p<0.05 (significance between different genotype, same treatment); $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 (significance between different treatment, same genotype); °p<0.05 (significance between same treatment, same genotype, different mouse line) .

Additionally, I measured the locomotor activity in the already addicted state on the third day of conditioning in order to see hyperlocomotion effects in the animals. The results revealed

significant changes in both mouse lines. Cocaine treated (10 mg/kg) wild-type animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line significantly walked more centimetres compared to their saline-treated littermates (T_{10}=3.379, p=0.0070; Tab. 6). The same hyperlocomotion effect was found for the cocaine treated knock-out animals of this line compared to their saline controls (T_{10}=3.674, p=0.0043; Tab. 4). Cocaine treated (10 mg/kg) mice of the J-CB1$^{-/-}$ mouse line showed the same hyperlocomotion effect. Wild-type animals of this line significantly increased the distance moved compared to their saline treated mice (T_{13}=2.609, p=0.0216; Tab. 6 and 7). Additionally, this effect was seen for the knock-out animals of J-CB1$^{-/-}$. J-CB1$^{-/-}$ knock-out mice under cocaine treatment significantly moved more centimetres than their respective animals of same genotype but under saline treatment (T_{11}=7.693, p<0.0001; Tab. 6 and 7). By comparison of the drug effect on both different genotypes it appeared that the knock-out animals of J-CB1$^{-/-}$ under cocaine treatment significantly increased even more their distance moved than their respective wild-type littermates (T_{13}=2.609, p=0.0216; Tab. 6). It was found as well that cocaine treated (10 mg/kg) knock-out animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line compared to the cocaine treated (10 mg/kg) knock-out animals of J-CB1$^{-/-}$ line showed a significant decrease in distance moved (T_{12}=2.896, p=0.0134; Tab. 6).

	J-CB1$^{-/-}$ (10 mg/kg cocaine)				**J-CB1$^{-/-}$ (20 mg/kg cocaine)**			
CPP	**+/+**		**-/-**		**+/+**		**-/-**	
group	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**
pre test	**Distance moved (cm)**							
all compartments	8065 ± 287.8		8055 ± 380.1		7997 ± 171.2		8572 ± 339.1	
black compartment	3360 ± 125.4		3455 ± 150.2		3458 ± 118.2		3687 ± 170.7	
white compartment	2624 ± 142.0		2723 ± 115.7		2618 ± 111.2		2934 ± 151.1	
group	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**	**saline**	**cocaine**
3.conditioning	**Distance moved (cm)**							
drug-associated compartment	2954 ± 237.1	3849 ± 201.1$^{\#;\circ\circ\circ}$	2195 ± 278.1	4525 ± 166.8$^{*,\#\#\#}$	3226 ± 310.5	7696 ± 704.2$^{\#\#\#}$	2884 ± 169.9	9604 ± 1417$^{\#\#;\circ\circ\circ}$

Table 7: Locomotion differences caused by cocaine (10 and 20 mg/kg). Evaluation of locomotion (distance moved), in naïve state and during 20 min conditioning in J-CB1$^{-/-}$ null-mutant mouse line, treated with two different doses of cocaine; +/+ (wild-type), -/- (knock-out); t-test analysis: *p<0.05 (significance between different genotype, same treatment; same dose); $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 (significance between different treatment, same genotype); $^{\circ\circ}$p<0.01 and $^{\circ\circ\circ}$p<0.001 (significance between same treatment, same genotype, different dose).

Next, I compared the locomotor activity of CB1 null-mutant line after treatment with two different doses of cocaine (10 and 20 mg/kg). The results are presented in Tab. 7. In the same

manner to the results with 10 mg/kg cocaine, the naïve animals of J-CB1$^{-/-}$ did not display a change in the distance moved compared with their respective wild-type littermates (T_{26}=1.513, p=0.1424); Tab. 7). Obviously, cocaine treatment (20 mg/kg) of the J-CB1$^{-/-}$ mice showed a similar hyperlocomotion effect as compared to the results gained with the 10 mg/kg dose. Wild-type littermates significantly increased the distance moved compared to their saline treated mice (T_{12}=5.179, p=0.0002; Tab. 6). This hyperlocomotion effect was found in the knock-out animals as well. Cocaine treated (20 mg/kg) J-CB1$^{-/-}$ knock-out mice significantly moved more in addicted state compared to their respective animals of same genotype but under saline treatment (T_{12}=4.048, p=0.0016; Tab. 6). The effect of the higher dose on genotypes revealed that the knock-out animals of J-CB1$^{-/-}$ injected with 20 mg/kg cocaine significantly increased their distance moved than the knock-out animals treated with 10 mg/kg (T_{14}=3.558, p=0.0031; Tab. 6). This effect was obvious in the wild-type littermates of J-CB1$^{-/-}$ as well. Treatment with 10 mg/kg cocaine significantly decreased the distance moved in J-CB1$^{-/-}$ wild-type animals compared to their respective genotype, treated with 20 mg/kg cocaine (T_{13}=4.937, p=0.0003; Tab. 6)

5.1.5.2 Conditioned place aversion test

Conditioned place aversion (CPA) test is another model of addiction, which provides data that can be used to substantiate results of CPP tests. By using this model I liked to recapitulate the results of the conditioned place preference test made with both C57BL/6 backgrounds. Both C57BL/6J and C57BL/6N were tested once in this paradigm by use of two different nicotine doses and saline as vehicle control.

Fig. 62 shows the results of the CPA with C57BL/6N mice. Blocking the chronic nicotine treatment with the nicotine antagonist mecamylamine (3.5 mg/kg) decreased the time spent in the drug-associated compartment for both used nicotine doses, but this effect was only significant for the nicotine dose of 10 mg/kg/d (T_{13}=2.269; p=0.0409), but not for the 36 mg/kg/d dose (T_{13}=1.832; p=0.0900). Mecamylamine injection after chronic vehicle treatment was not significantly changing time spent in the drug-associated compartment (T_8=1.565; p=0.1563).

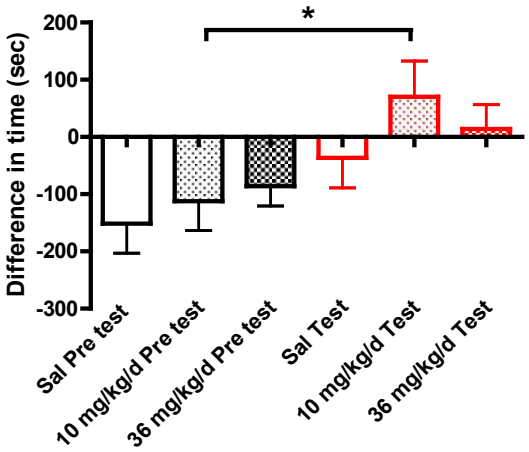

Fig. 62: Effect of mecamylamine (3.5 mg/kg) injections on the time spent in the drug-associated compartment in C57BL/6N animals. Blockade of chronic nicotine/vehicle treatment decreased the time spent in the drug-associated compartment in all groups. But significance only appeared for the 10 mg/kg nicotine group. *p< 0.05 (t test; n=8/nicotine group and n=5/vehicle group, male animals, 10-12 weeks old).

The results of the CPA test with C57BL/6J animals are shown in Fig. 63. In this experiment the blockade of the chronic nicotine treatment with the nicotine antagonist mecamylamine (3.5 mg/kg) significantly decreased the time spent in the drug-associated compartment in both the 10 mg/kg/d (T_{16}=4.121; p=0.0008) and the 36 mg/kg/d dose (T_{16}=6.193; p<0.0001). It can be seen that the vehicle group exhibits a decrease in the time spent in drug-associated compartment as well. But this decrease is not significant (T_6=1.416; p=0.2064).

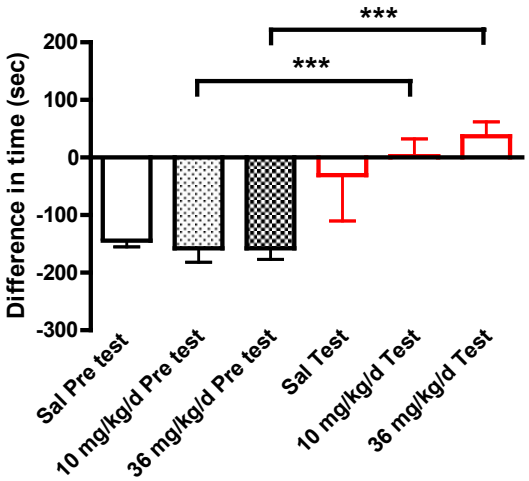

Fig. 63: Effect of mecamylamine (3.5 mg/kg) injections in decreasing the time spent in the drug-associated compartment in C57BL/6J animals. Blockade of chronic nicotine/vehicle treatment decreased the time spent in the drug-associated compartment in all groups. But this effect was not significant in the saline receiving group. ***p<0.001 (t test; n=9/nicotine group and n=4/vehicle group, male animals, 4 months old).

5.2. Molecular analyses of CB1$^{f/f;D1\text{-}Cre}$ mouse line

5.2.1. Analyses of gene expression in striatum of CB1$^{f/f;D1\text{-}Cre}$ mice after nicotine treatment

5.2.1.1 GeneChip Mouse Genome 430A 2.0 Array

The GeneChip® Mouse Genome 430A 2.0 Array is a single array that contains over 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes that can be used to explore mechanisms underlying biological and disease processes. Sequences used in the design of the array were selected from GenBank®, dbEST, and RefSeq. With the help of the gene array, I evaluated the gene expression changes in the striatum of the mutant line CB1$^{f/f;D1\text{-}Cre}$ after a conditioned place preference (CPP) experiment with repeated injections of 0.5 mg/kg nicotine. For each group (Tab. 3), 22,600 probe sets representing transcripts and variants from over 14,000 well-characterized mouse genes were tested. The analysis of raw Affymetrix gene array data was performed at the Institute for Translational Oncology (TRON). Bioinformatics processed the microarray data of all 22 Affymetrix microarrays by the RMA algorithm (Irizarry et al., 2003) implemented in the Bioconductor R package (Gentleman et al., 2004; R Development Core Team, 2010). For this, custom probe annotation was added (Dai et al., 2005). For comparison of the expression data, the R package limma (Smyth, 2004) was used to estimate fold changes for the defined contrasts and calculate false discovery rates (FDRs) by the Benjamini and Hochberg method (1995). Additionally, the raw data of the gene array was analysed with special Partek Genomics Suite program at the TRON institute. Pathway analyses were made with the IPA ® Ingenuity Pathway analyses software (www.ingenuity.com). These analyses were based only on p-value, not FDR value. Out of the high amount of raw data, the 15 strongest up- or downregulated genes were filtered (s. Appendix of this thesis) and the results were compared with the genes from analysis with the Partek Genomics Suite program. Out of this comparison, nine candidate genes were chosen (Tab. 8), the gene *cnr1* coding for the CB_1 receptor as a positive control.

Symbol	Publicised gene name	Location of protein	Type
pttg1	pituitary tumor transforming 1	nucleus	transcription regulator
nptx1	neuronal pentraxin 1	extracellular space	other
nts	neurotensin	extracellular space	other
klf4	Kruppel-like factor 4 (gut)	nucleus	transcription regulator
tbr1	T-box, brain, 1	nucleus	transcription regulator
ttr	transthyretin	extracellular Space	transporter
egr2	early growth response 2	nucleus	transcription regulator
neurod6	neurogenic differentiation 6	nucleus	other
calml4	calmodulin-like 4	unknown	other

Table 8: Candidate genes. Evaluation of the gene expression results of the GeneChip® Mouse Genome 430A 2.0 Array lead to the candidate genes, which showed the strongest differences in the fold changes.

5.2.1.2 Fold changes of candidate genes based on treatment and behaviour of tested animals

First I compared the basal phenotype of untreated animals of both genotypes of naïve $CB1^{f/f;D1\text{-}Cre}$ animals. In Fig. 64 I visualized the different fold changes (FC) of the candidate genes. The control gene *cnr1* showed the expected two to three fold upregulation in wild-type animals compared to their respective knock-out littermates (FC = 3.0474). A positive fold change was found for *pttg1* (8.6555), *klf4* (3.034899695), *tbr1* (1.1265), *egr2* (4.1096) and *neurod6* (1.5200) as well. The fold change difference of the other candidate genes, *nptx1* (-2.2957), *nts* (-5.5264), *ttr* (-96.9988) and *calml4* (-10.8380) revealed a downregulation.

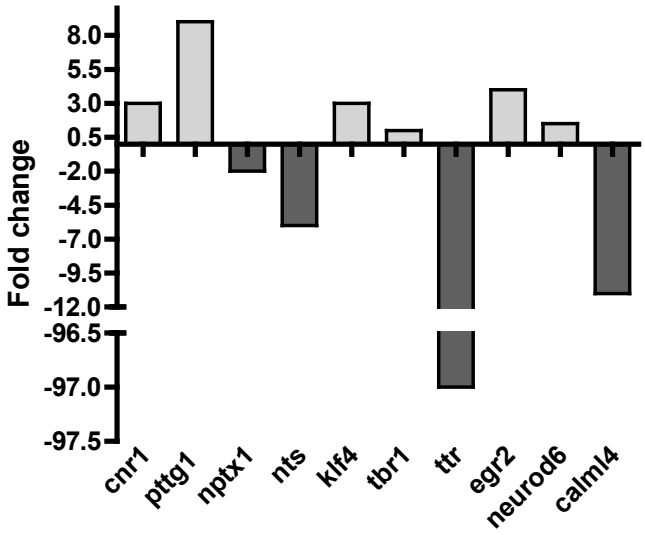

Fig. 64: Fold changes of control and candidate genes in basal phenotype. Expression differences of naïve untreated animals of $CB1^{f/f;D1\text{-}Cre}$ wild-type mice compared to their respective knock-out littermates (n=3/group; male animals, 6-7 months old)

Next, I estimated how the effect of vehicle treatment changed the phenotype of both genotypes of CB1$^{f/f;D1\text{-}Cre}$ mice. Fig. 65 presents the effect of saline injections on the fold changes (FC) of the candidate genes in wild-type animals, while the same effect in knock-out mice is shown in Fig. 65. Our control gene *cnr1* showed in both figures no difference in fold change (Fig. 64 FC=1.0108; Fig. 65 FC=-1.4440) expected for groups of the same genotype. The results for the vehicle-induced effect on wild-type animals presented positive fold changes for the candidate genes *nts* (3.0571), *klf4* (1.4240) and *egr2* (2.2660) while *pttg1* (-4.5530) and *nptx1* (-2.2957) showed a negative fold change. The genes *tbr1* (1.1437), *ttr* (1.2534) *neurod6* (-1.0009) and *calml4* (1.1064) presented no expression changes in untreated and saline injected wild-types of CB1$^{f/f;D1\text{-}Cre}$ mice.

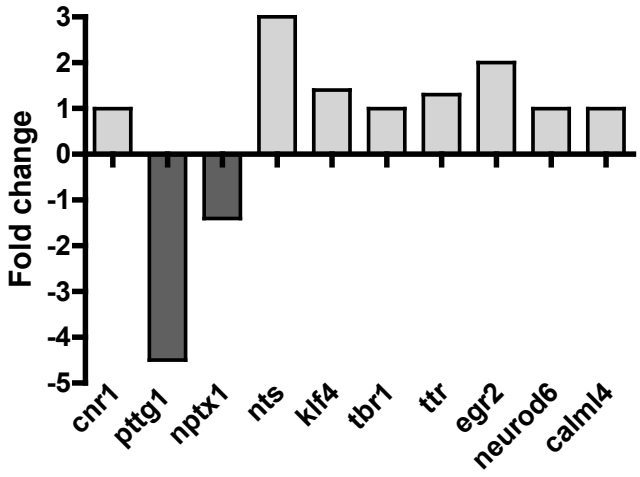

Fig. 65: Fold changes of control and candidate genes in the stress-induced phenotype. Expression differences of untreated CB1$^{f/f;D1\text{-}Cre}$ wild-type mice compared to their respective saline injected wild-type littermates (n=3/group; male animals, 6-7 months old)

Additionally, knock-out animals of this conditional mutant line were compared (Fig. 66). The results for the vehicle-induced effect revealed positive fold changes for the candidate genes *pttg1* (274.3304), *nts* (14.8786), *ttr* (33.8070) and *calml4* (32.4081). *klf4* (-3.7419), *tbr1* (-1.4898), *egr2* (-3.1698) and *neurod6* (-1.4683) showed a negative fold change. Only the gene *nptx1* (1.1240) showed no expression changes in untreated versus saline injected knock-out animals.

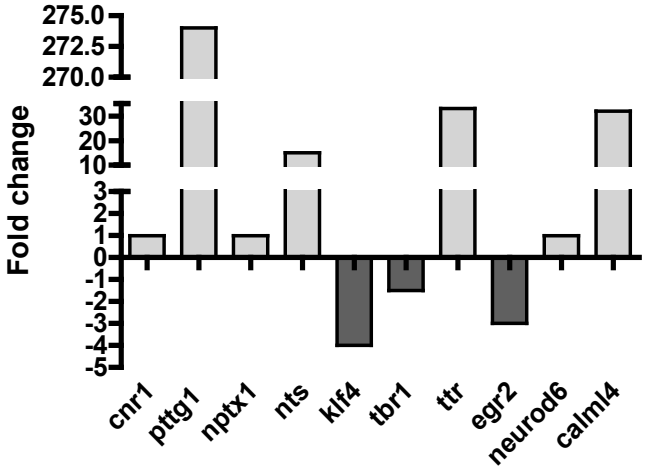

Fig. 66: Fold changes of control and candidate genes in the stress-induced phenotype. Expression differences of naïve, untreated $CB1^{f/f;D1\text{-}Cre}$ knock-out animals compared to their respective saline injected knock-out littermates (n=3/group; male animals, 6-7 months old)

By comparison of vehicle treated animals of both genotypes (Fig. 67), I could show that he candidate gene *pttg1* (10756.0336) manifested a strong upregulation in wild-type mice compared to knock-out animals. Additionally, a positive fold change could be seen for *calml4* (2.7027). The genes *nts* (-1.1355), *tbr1* (-1.1564) and *neurod6* (1.0477) showed no expression changes, while *nptx1* (-1.4541), *klf4* (-1.7557), *ttr* (-3.5963) and *egr2* (-1.7478) revealed a negative fold change. The control gene *cnr1* exhibited the expected two till three fold upregulation in wild-type animals compared to their respective knock-out littermates (FC=2.0865).

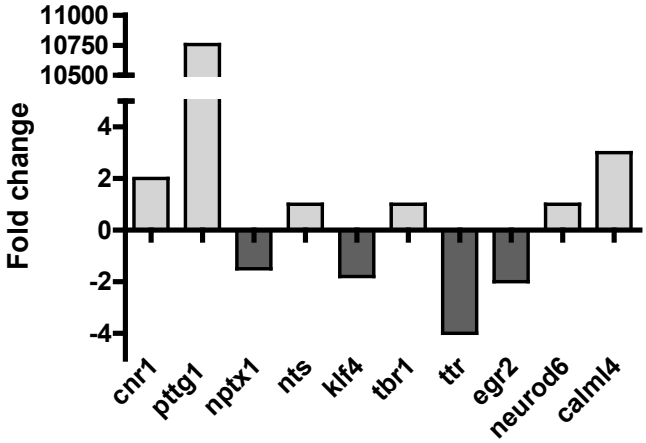

Fig. 67: Fold changes of control and candidate genes after vehicle treatment in both genotypes. Expression differences of saline treated $CB1^{f/f;D1\text{-}Cre}$ wild-type animals compared to their respective saline injected knock-out littermates (n=3/group; male animals, 6-7 months old)

Next, I aimed to evaluate the effect of repeated nicotine injections (0.5 mg/kg) on the different behaviour seen in wild-type and knock-out animals of the conditional mutant CB1$^{f/f;D1\text{-}Cre}$ mouse line. I compared groups of different genotype, which showed the same behaviour in the post test of a conditioned place preference (CPP) experiment. In Fig. 68 the results for the nicotine effect in both genotypes, in developing preference, were visualized. As expected, the control gene was not effected by nicotine injections and showed only the genotype effect (FC=2.6719). An effect of nicotine on gene regulation in the striatum of wild-type animals was visible in the positive fold change for the candidate genes *klf4* (2.8902) and *egr2* (1.8159). *Pttg1* (-21.5767) and *calml4* (-4.3870) showed a negative fold change. The candidate genes *nptx1* (-1.1081), *nts* (-1.0105), *tbr1* (1.1437), *ttr* (1.0874) and *neurod6* (1.0664) presented no expression changes in both genotypes of CB1$^{f/f;D1\text{-}Cre}$ animals, injected four times with 0.5 mg/kg nicotine.

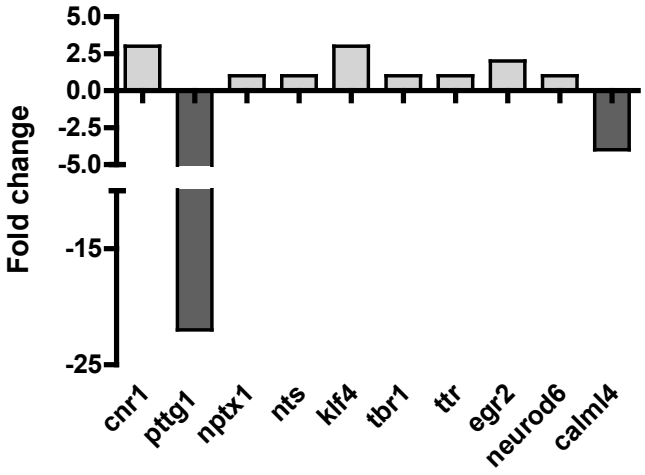

Fig. 68: Fold changes of control and candidate genes after nicotine treatment (0.5 mg/kg) in both genotypes. Expression differences of nicotine injected CB1$^{f/f;D1\text{-}Cre}$ wild-type animals compared to their respective nicotine treated knock-out littermates, which exhibited preference in the conditioned place preference paradigm (n=3/group; male animals, 6-7 months old)

In Fig. 69, the visualization of the nicotine effect is presented in developing aversion in both genotypes. Analogous to Fig. 67, the control gene was not effected by nicotine injections and only the genotype effect (FC=2.8509) was recognisable. The effect of repeated nicotine injections on the gene regulation of wild-type compared to knock-out animals is shown in the positive fold change for the candidate genes *pttg1* (9505.1769) and *egr2* (1.7983). *Klf4* (-1.1792) and *calml4* (1.1455) showed no difference in fold change. The candidate genes *nptx1* (-8.2770), *nts* (-11.4312), *tbr1* (-6.6863), *ttr* (-28.8181) and *neurod6* (-37.4585) presented negative fold changes in both genotypes of CB1$^{f/f;D1\text{-}Cre}$ animals.

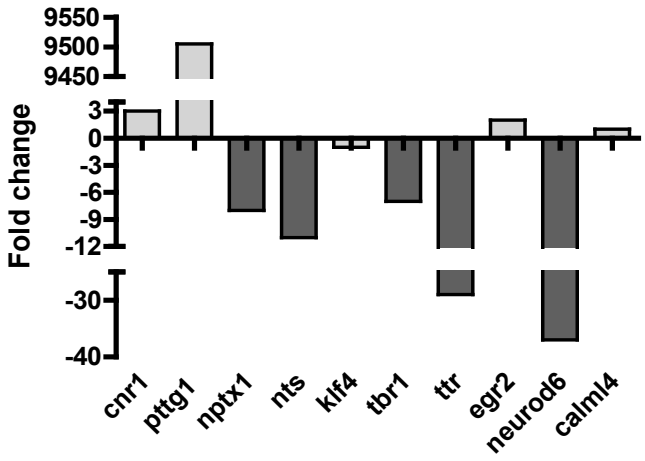

Fig. 69: Fold changes of control and candidate genes after nicotine treatment (0.5 mg/kg) in both genotypes. Expression differences of nicotine injected $CB1^{f/f;D1\text{-}Cre}$ wild-type animals compared to their respective nicotine treated knock-out littermates, which exhibited aversion in the conditioned place preference experiment (n=3/group; male animals, 6-7 months old)

Thereupon, I wanted to have a closer look on the effect of repeated nicotine injections (0.5 mg/kg) on the different behaviour seen in the same genotype. So I compared the groups of same genotype, which showed a variant behaviour in the post test of the CPP experiment.

In Fig. 70 the results for the nicotine effect in wild-type mice are shown, presented by the differences of untreated animals compared to nicotine treated animals, which showed preference in the post test. The control gene *cnr1* did not change (1.0896) as expected for mice of the same genotype. A positive fold change for the candidate genes was only to recognize for *pttg1* (915.2770) and *calml4* (1.7555). The genes *nts* (-2.0867), *klf4* (-2.0179) and *egr2* (-2.9311) exhibited a negative fold change. The candidate genes *nptx1* (-1.0125), *tbr1* (1.1711), *ttr* (1.1058) and *neurod6* (1.2527) presented no expression changes in the different treated animals of wild-type $CB1^{f/f;D1\text{-}Cre}$ mice.

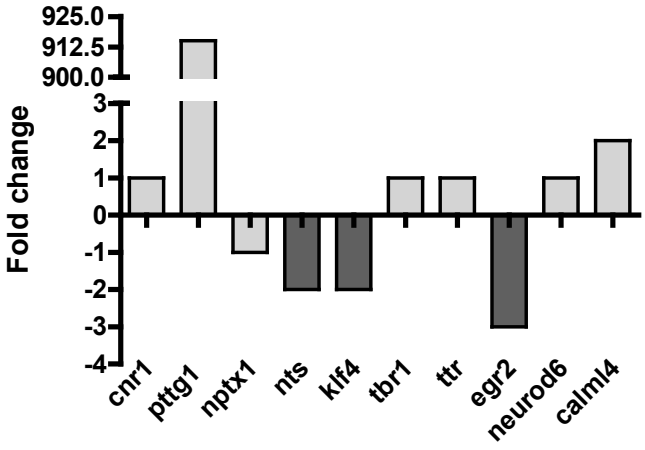

Fig. 70: Fold changes of control and candidate genes after vehicle or nicotine treatment (0.5 mg/kg) in wild-type CB1$^{f/f;D1\text{-}Cre}$ mice. Expression differences of saline injected CB1$^{f/f;D1\text{-}Cre}$ wild-type animals compared to their respective nicotine treated wild-type littermates, which exhibited preference in the CPP experiment (n=3/group; male animals, 6-7 months old)

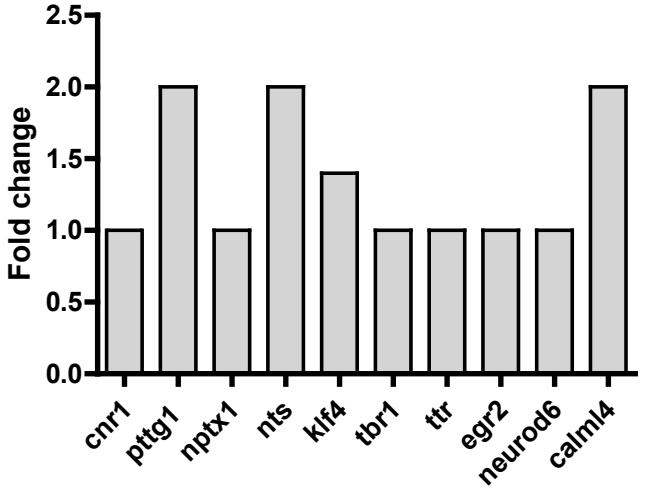

Fig. 71: Fold changes of control and candidate genes after vehicle or nicotine treatment (0.5 mg/kg) in wild-type CB1$^{f/f;D1\text{-}Cre}$ mice. Expression differences of saline injected CB1$^{f/f;D1\text{-}Cre}$ wild-type animals compared to their respective nicotine treated wild-type littermates, which exhibited aversion in the CPP experiment (n=3/group; male animals, 6-7 months old)

As seen in Fig. 71, the results for the nicotine effect in wild-type mice are visualized, this time presented by the differences of untreated animals compared to nicotine treated animals, which showed aversion in the post test. As mentioned before the control gene *cnr1* did not express a fold change (1.0514) between the groups of the same genotype. Only four candidate genes exhibited positive fold change, *pttg1* (1.8771), *nts* (1.7611), *klf4* (1.3144) and

calml4 (1.9643). The candidate genes *nptx1* (1.1487), *tbr1* (1.1483), *ttr* (1.0001), *egr2* (1.2158) and *neurod6* (1.2117) presented no expression changes in the different treated animals of wild-type CB1$^{f/f;D1\text{-}Cre}$ mice with different behaviour expression.

For the wild-type group, I compared those animals, which developed preference, with the ones showing aversion after nicotine treatment (Fig. 72). Again, the control gene *cnr1* did not change (-1.0363) between the groups of the same genotype. In this comparison three candidate genes showed a positive fold change, *nts* (3.6749), *klf4* (2.6522) and *egr2* (3.5636), while *pttg1* (-509.2579) revealed a strong negative fold change. The candidate genes *nptx1* (1.2642), *tbr1* (-1.0199), *ttr* (-1.1057), *neurod6* (-1.0338) and *calml4* (1.1189) presented no expression changes in the nicotine treated animals of wild-type CB1$^{f/f;D1\text{-}Cre}$ mice with different behaviour expression.

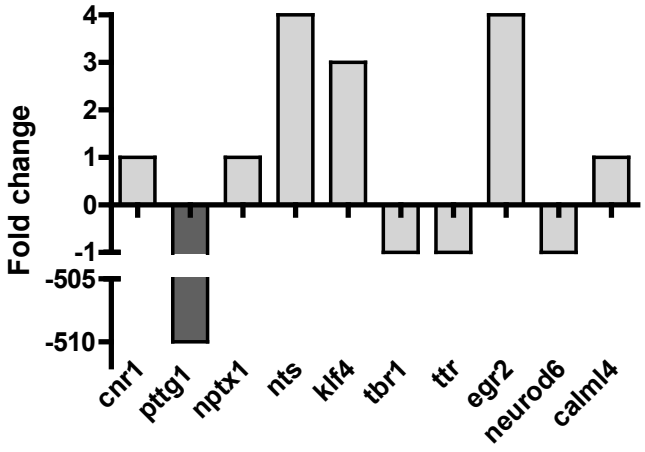

Fig. 72: Fold changes of control gene and candidate genes after nicotine treatment (0.5 mg/kg) in wild-type CB1$^{f/f;D1\text{-}Cre}$ mice. Expression differences of nicotine injected, preference showing CB1$^{f/f;D1\text{-}Cre}$ wild-type animals compared to their respective nicotine treated wild-type littermates, which showed aversion in the CPP experiment (n=3/group; male animals, 6-7 months old)

Analogue to the comparisons made for wild-type mice of CB1$^{f/f;D1\text{-}Cre}$ animals, I analysed the fold changes for the knock-out mice of this mouse line as well. Fig. 73 presents the results for the nicotine effect in CB1$^{f/f;D1\text{-}Cre}$ knock-out mice. This effect is depicted by the differences of saline-treated animals compared to nicotine treated animals, which were showing preference behaviour in the post test of the CPP experiment before. The fold change of control gene *cnr1* (1.3953) did not reveal a difference, which should be the case for animals of the same genotype. A positive fold change for the candidate genes was visible for *klf4* (2.5148), *tbr1* (1.4726) and *ttr* (3.2075), while *pttg1* (-33.9364), *nts* (-1.8569) and *calml4* (-6.7542) revealed a negative fold change. The genes *nptx1* (1.2960), *egr2* (1.0828) and *neurod6* (1.2787) presented no expression changes in the different treated CB1$^{f/f;D1\text{-}Cre}$ knock-out mice.

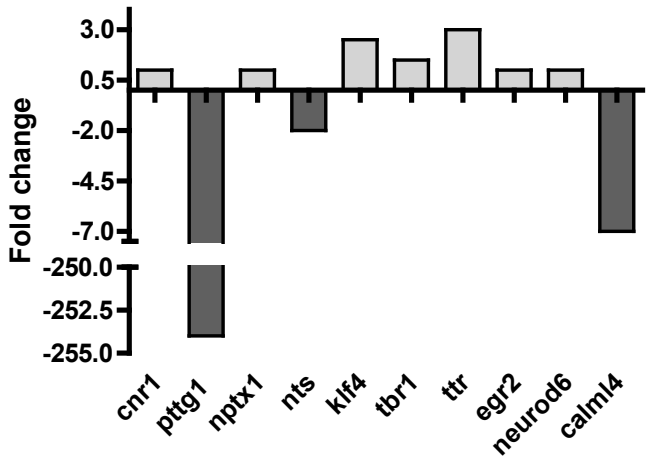

Fig. 73: Fold changes of control and candidate genes after vehicle or nicotine treatment (0.5 mg/kg) in knock-out CB1$^{f/f;D1\text{-}Cre}$ mice. Expression differences of saline injected CB1$^{f/f;D1\text{-}Cre}$ knock-out animals compared to their respective nicotine treated knock-out littermates, which exhibited preference in the CPP experiment (n=3/group; male animals, 6-7 months old)

Additionally, saline-treated animals were compared to nicotine treated animals, which showed aversion in the post test. The results of this comparison can be seen in Fig. 74. Again, the control gene *cnr1* did not change (1.4366) between the groups of the same genotype. A positive fold change was apparent for the three candidate genes *pttg1* (1.9961), *klf4* (1.9569) and *egr2* (3.8214). *Calml4* (-1.2012) presented no expression changes, indicating no nicotine effect in the different treated animals of knock-out CB1$^{f/f;D1\text{-}Cre}$ mice with different behaviour expression. But the candidate genes *nptx1* (-4.5585), *nts* (-5.7163), *tbr1* (-5.0355), *ttr* (-8.0127) and *neurod6* (-32.2963) showed a gene upregulation after repeated nicotine treatment.

Next, I compared knock-out animals of the same treatment, but with a different behaviour in response to this treatment. Mice, which developed preference, were matched with their littermates, which showed aversion after nicotine treatment (Fig 75). For the control gene *cnr1* no difference in the fold change (1.0296) was found. In this comparison positive fold changes could be seen for *pttg1* (402.7232), *egr2* (3.5292) and *calml4* (5.6230). The candidate genes *nptx1* (-5.9079), *nts* (-3.0784), *tbr1* (-7.4155), *ttr* (-25.7008) and *neurod6* (-41.2976) revealed negative fold changes. Only the expression levels of *klf4* (-1.2851) were unchanged in the nicotine treated knock-out mice of CB1$^{f/f;D1\text{-}Cre}$ mouse line with different behaviour expressions.

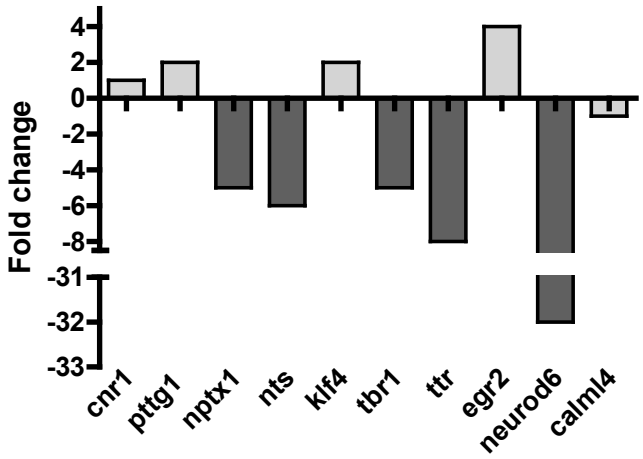

Fig. 74: Fold changes of control and candidate genes after vehicle or nicotine treatment (0.5 mg/kg) in knock-out mice of CB1$^{f/f;D1\text{-}Cre}$ mouse line. Expression differences of saline injected CB1$^{f/f;D1\text{-}Cre}$ knock-out animals compared to their respective nicotine treated knock-out littermates, which developed aversion in the CPP experiment (n=3/group; male animals, 6-7 months old)

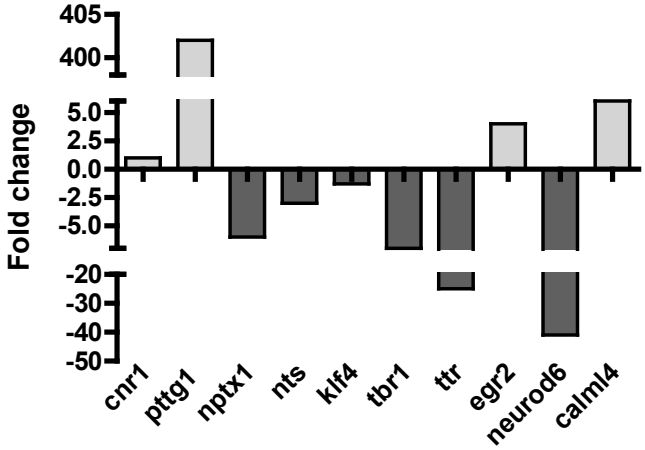

Fig. 75: Fold changes of control gene and candidate genes after nicotine treatment (0.5 mg/kg) in knock-out CB1$^{f/f;D1\text{-}Cre}$ mice. Expression differences of nicotine injected, preference showing CB1$^{f/f;D1\text{-}Cre}$ knock-out animals compared to their respective nicotine treated knock-out littermates, which developed aversion behaviour in the CPP experiment (n=3/group; male animals, 6-7 months old)

Last, I was interested to know, how the effect of repeated nicotine injections (0.5 mg/kg) looks on the different behaviour seen in both genotypes. Therefore, I compared wild-type animals of CB1$^{f/f;D1\text{-}Cre}$, which showed preference behaviour in the post test of conditioned place preference experiment, with knock-out animals, which developed aversion in the same experiment. Fig. 76 reveals the results of this comparison. The control gene *cnr1* showed the

known fold change (2.7509), as expected for mice of the different genotype. A positive fold change for the candidate genes was recognized for *pttg1* (7.9912), *klf4* (2.2491) and *egr2* (6.4086). A negative fold change could be found for the candidate genes *nptx1* (-6.5468), *nts* (-3.1106), *tbr1* (-6.8193), *ttr* (-31.8650) and *neurod6* (-38.7259). Only *calml4* (1.2817) presented no expression changes in the nicotine treated animals of wild-type and knock-out $CB1^{f/f;D1\text{-}Cre}$ mice.

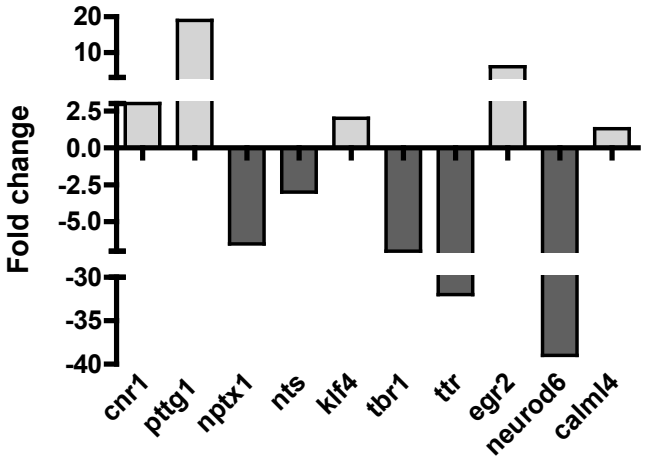

Fig. 76: Fold changes of control gene and candidate genes after nicotine treatment (0.5 mg/kg) in $CB1^{f/f;D1\text{-}Cre}$ mice. Expression differences of nicotine injected, preference showing $CB1^{f/f;D1\text{-}Cre}$ wild-type animals compared to their respective nicotine treated knock-out littermates, which showed aversion behaviour in the post test of CPP experiment (n=3/group; male animals, 6-7 months old)

5.2.2. Gene expression analyses of candidate genes from GeneChip® Mouse Genome 430A 2.0 Array by quantitative real-time polymerase chain reaction in the $CB1^{f/f;D1\text{-}Cre}$ mouse line

The candidate genes, chosen from the GeneChip® Mouse Genome 430A 2.0 Array were analysed by quantitative real-time polymerase chain reaction (qPCR) by using TaqMan® MGB probes from Applied Biosystems. Results were processed and analysed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) as it is important that statistical data is converted to the linear form by the $2^{-\Delta\Delta CT}$ calculation and is not presented by the raw CT values.

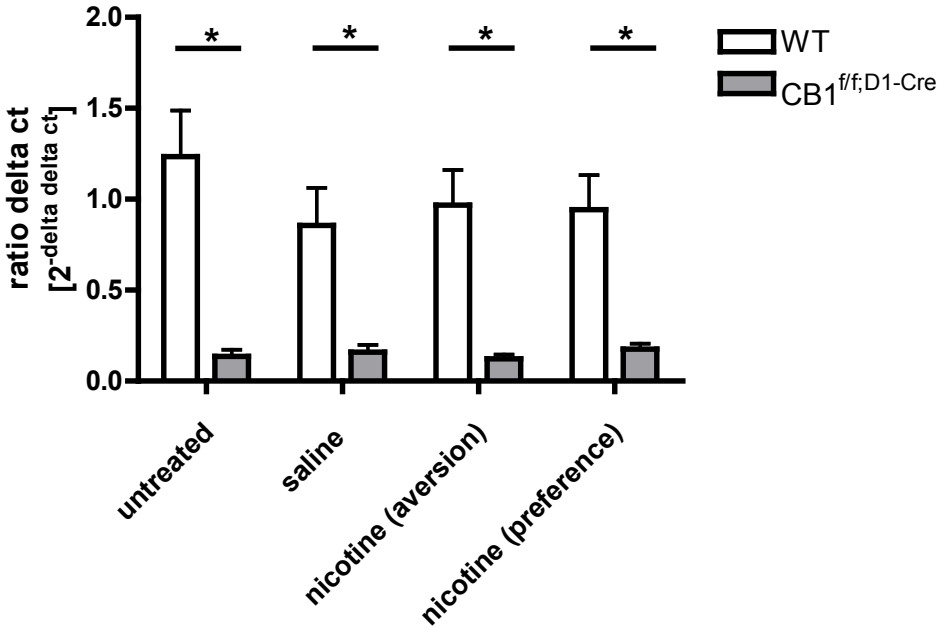

Fig. 77: Relative gene expression changes for the control gene *cnr1* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ mice in different states of treatment. Knock-out CB1$^{f/f;D1\text{-}Cre}$ mice of all different groups display a significantly decreased gene expression compared to their respective wild-type (WT) littermates. *p<0.05 (t test; n=3/group; male animals, 6-7 months old)

The results of the qPCR for the control gene *cnr1* are presented in Fig. 77. It can be seen that CB1$^{f/f;D1\text{-}Cre}$ mice showed a significantly lower *cnr1* expression compared to their respective wild-type independent of the treatment. The ratio of ΔCT for the untreated CB1$^{f/f;D1\text{-}Cre}$ mice was 0.1353 ± 0.03667 compared to the ratio of ΔCT of 1.234 ± 0.2532 of untreated wild-type littermates. Analysed by student´s t test, this difference was significant (T_4=4.295; p=0.0127). Vehicle treated mice showed the same effect (T_4=3.320; p=0.0294). The treatment with 0.5 mg/kg nicotine during the CPP experiment had no different effect on gene expression of *cnr1*. Nicotine treated animals, which expressed aversion in post test of the CPP experiment displayed as well a significant gene expression change for *cnr1* (T_4=4.328; p=0.0124). This could additionally be found for both nicotine treated genotypes of CB1$^{f/f;D1\text{-}Cre}$ animals (T_4=3.969; p=0.0166). Two way ANOVA detected that these effects in the different groups were not significant compared to each other for treatment ($F_{3,16}$=0.51; p=0.6800).
Fig. 78 shows the qPCR results for the two candidate genes *pttg1* (Fig. 78A) and *nptx1* (Fig. 78B) in different states of treatment. For *pttg1* (Fig. 78A) only a significant difference in the ratio of ΔCT could be found for the control group of vehicle treated animals. CB1$^{f/f;D1\text{-}Cre}$ mice differed significantly from their wild-type littermates (T_4=3.882; p=0.0178).

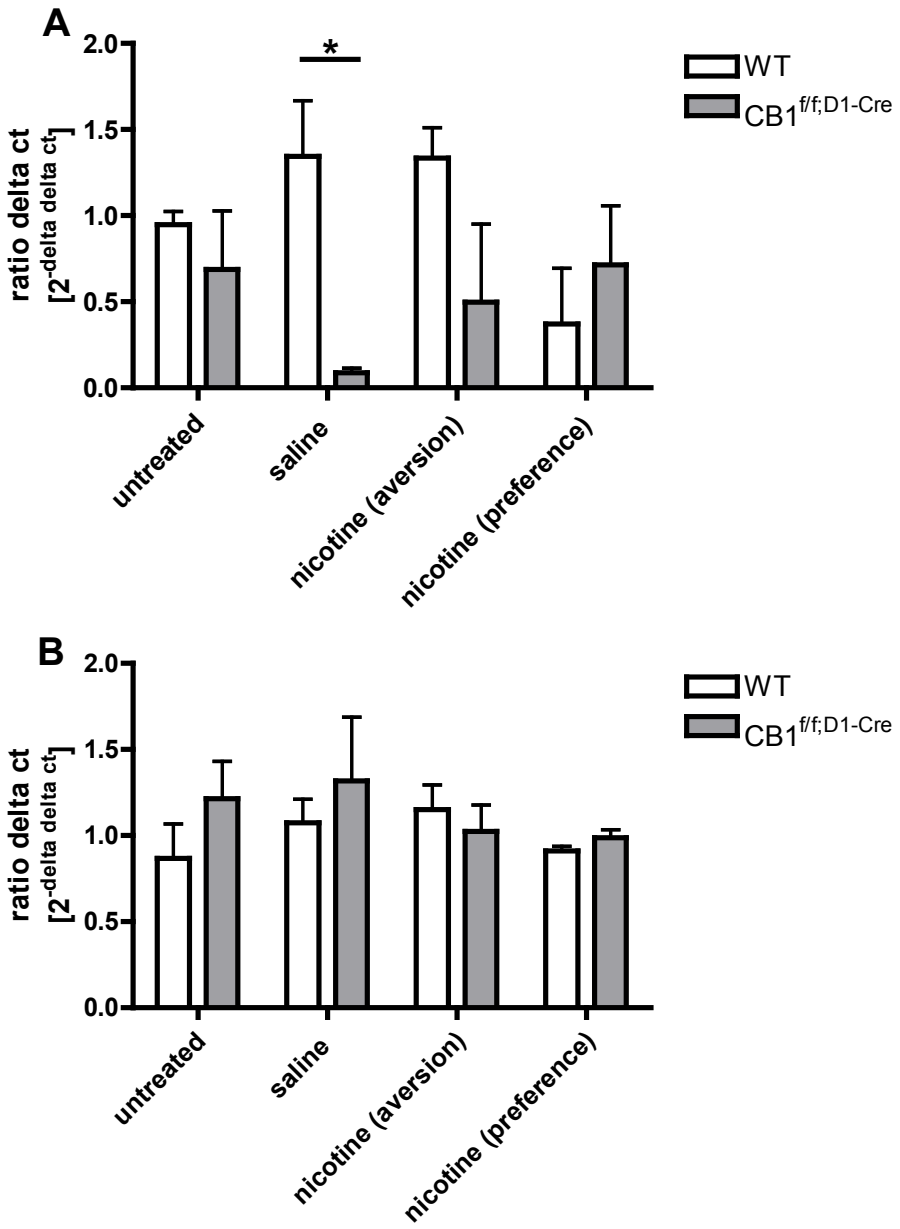

Fig. 78: Relative gene expression changes for the candidate genes *pttg1* (A) and *nptx1* (B) in the striatum of CB1$^{f/f;D1\text{-}Cre}$ mice in different states of treatment. (A) Knock-out CB1$^{f/f;D1\text{-}Cre}$ mice of saline treated control group showed a significantly decreased gene expression of *pttg1* compared to their respective wild-type littermates. (B) No significant gene expression changes for *nptx1* were found in all groups. *p<0.05 (t test; n=3/group; male animals, 6-7 months old)

But post hoc analyses did not reveal any additional, significant change in gene expression for the other groups (untreated group, WT vs. CB1$^{f/f;D1\text{-}Cre}$ mice T_4=0.7453; p=0.4975; nicotine (aversion) group WT vs. CB1$^{f/f;D1\text{-}Cre}$ mice T_4=1.717; p=0.1611; nicotine (preference) group, WT vs. CB1$^{f/f;D1\text{-}Cre}$ mice T_4=0.7255; p=0.5083). Animals of the same genotype of each group did not differ in the gene expression of *pttg1* compared to each other (one way ANOVA, wild-

type mice F= 3.416; p=0.0731; CB1$^{f/f;D1\text{-}Cre}$ animals F= 0.7594; p=0.5476). Two way ANOVA did not find a significant effect for treatment ($F_{3,16}$=0.5931; p=0.6285) or for the interaction of treatment and genotype ($F_{3,16}$=2.827; p=0.0718). No significant differences in the ratio of ΔCT could be found for the candidate gene *nptx1* (Fig. 78B). ANOVA did not find a significant effect for neither genotype ($F_{1,16}$=1.02; p=0.3276) or treatment ($F_{3,16}$=0.58; p=0.6351).

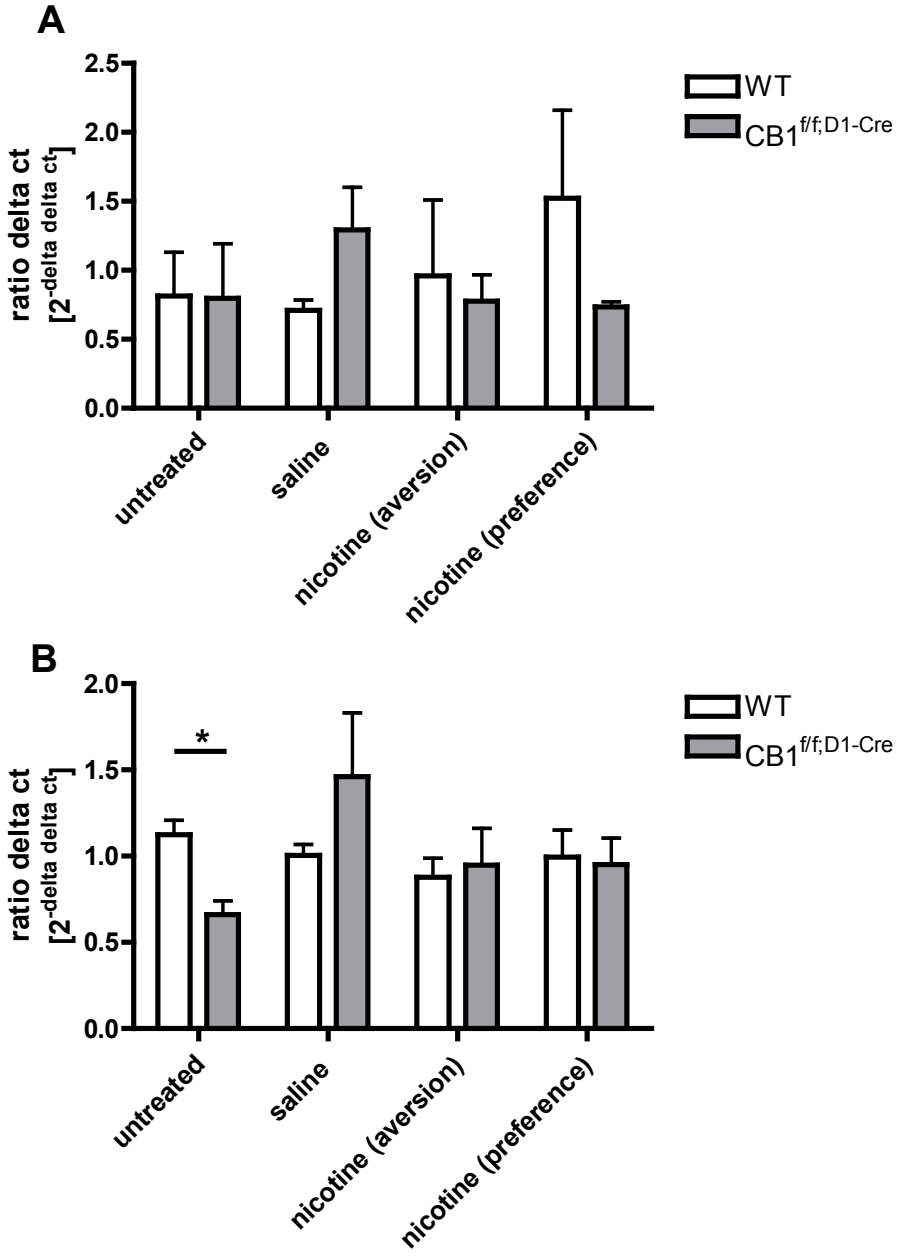

Fig. 79: Relative gene expression changes for the candidate genes *nts* (A) and *klf4* (B) in the striatum of CB1$^{f/f;D1\text{-}Cre}$ mice in different states of treatment. (A) No significant gene expression changes for *nts* were found in all groups. (B) Knock-out CB1$^{f/f;D1\text{-}Cre}$ animals of the untreated group showed a significantly decreased gene expression of *klf4* compared to their respective wild-type littermates. *p<0.05 (t test; n=3/group; male animals, 6-7 months old)

Next I evaluated the qPCR results for the two candidate genes *nts* (Fig. 79A) and *klf4* (Fig. 79B) in different states of treatment. No significant differences in the ratio of ΔCT could be detected for the candidate gene *nts* (Fig. 79A). ANOVA did not find a significant effect for genotype ($F_{1,16}$=0.15; p=0.7072), treatment ($F_{3,16}$=0.30; p=0.8253) or for the interaction of both factors ($F_{3,16}$=1.15; p=0.3581) on the regulation of this gene.

Analyses of the ratio of ΔCT for *klf4* (Fig. 79B) showed only a significant difference in the group of naïve, untreated animals. The *klf4* gene expression in $CB1^{f/f;D1\text{-}Cre}$ animals was significantly decreased compared to their wild-type littermates (T_4=4.008; p=0.0160), implicating a basal difference in these animals. Post hoc analyses for the gene regulation in the other groups did not reveal any additional, significant change in gene expression (vehicle treated group, WT vs. $CB1^{f/f;D1\text{-}Cre}$ mice T_4=1.216; p=0.2910; nicotine (aversion) group WT vs. $CB1^{f/f;D1\text{-}Cre}$ mice T_4=0.3011; p=0.7783; nicotine (preference) group, WT vs. $CB1^{f/f;D1\text{-}Cre}$ mice T_4=0.1922; p=0.8561). Comparisons of the mice of the same genotype of each group did not show differences in the gene expression of *klf4* (one way ANOVA, wild-type animals F= 0.8408; p=0.5087; $CB1^{f/f;D1\text{-}Cre}$ mice F= 2.084; p=0.1807). An additional two way ANOVA could not detect a significant effect for treatment ($F_{3,16}$=1.51; p=0.2499).

It was not possible, to visualize the results of the ratio of ΔCT for the candidate gene *ttr*, because of the extremely different ratios between the four groups. In all different states of treatment no significant changes in the ratio of ΔCT for *ttr* could be found. Unpaired t test with Welsh's correction showed a decreased gene expression for $CB1^{f/f;D1\text{-}Cre}$ mice compared to their wild-type littermates, but this decrease was not significant (T_2=1.017; p=0.4161). Contrarily, a decrease in gene expression could be detected for saline treated wild-type mice compared to $CB1^{f/f;D1\text{-}Cre}$ animals, but the effect was as well not significant (T_2=1.006; p=0.4202). For the nicotine treated animals, which presented an aversion in the post test of the CPP experiment, the ratio of ΔCT for *ttr* showed for $CB1^{f/f;D1\text{-}Cre}$ mice a decrease (0.7171 ± 0.7162) compared to the respective wild-type littermates (3.590 ± 3.580). But again, this decrease was not significant (T_2=0.7869; p=0.5138). The group of nicotine treated mice, revealing preference in the post test, had no differences at all (T_4=0.1281; p=0.9043). Animals of the same genotype of each group did not differ in the gene expression of *ttr* compared to each other (Kruskal-Wallis test, wild-type mice, Kruskal-Wallis statistic = 2.715; p=0.4377; $CB1^{f/f;D1\text{-}Cre}$ animals Kruskal-Wallis statistic = 2.897; p=0.4077). Additionally, two way ANOVA did not find a significant effect for treatment ($F_{3,16}$=0.70; p=0.5645) or genotype ($F_{1,16}$=0.10; p=0.7513).

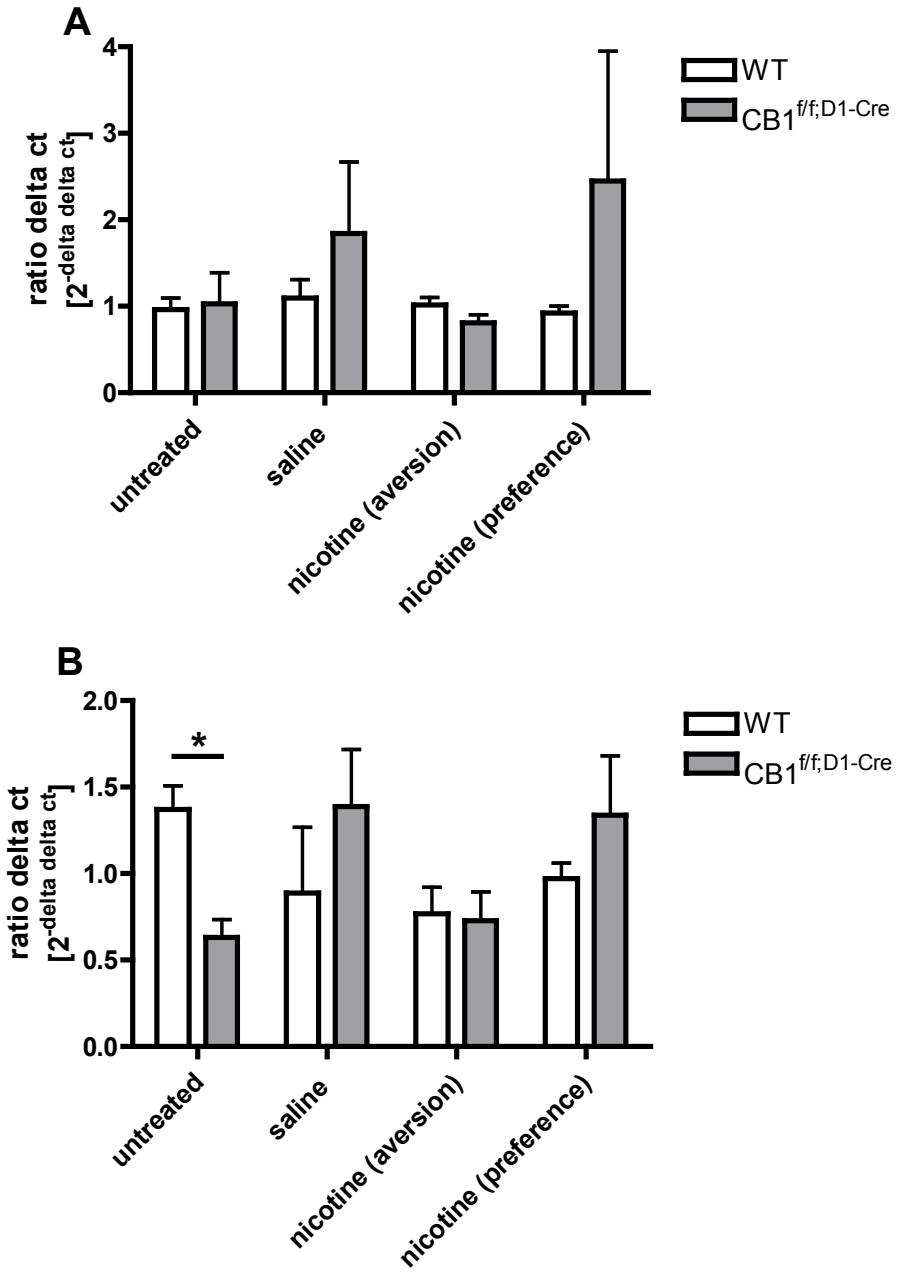

Fig. 80: Relative gene expression changes for the candidate genes *tbr1* (A) and *egr2* (B) in the striatum of CB1$^{f/f;D1\text{-}Cre}$ mice in different states of treatment. (A) No significant gene expression changes for *tbr1* were found in all groups. (B) Knock-out CB1$^{f/f;D1\text{-}Cre}$ animals of the untreated group showed a significantly decreased gene expression of *egr2* compared to their respective wild-type littermates. *p<0.05 (t test; n=3/group; male animals, 6-7 months old)

Fig. 80 presents the results of the relative gene expression changes for the candidate genes *tbr1* (A) and *egr2* (B) in the striatum of CB1$^{f/f;D1\text{-}Cre}$ mice in different states of treatment. No significant differences in the ratio of ΔCT could be detected for the candidate gene tbr1 (Fig. 80A). The increase of the ratio of ΔCT for saline treated and nicotine treated, preference

showing CB1$^{f/f;D1\text{-}Cre}$ mice compared to their respective wild-type animals showed also no significance (saline group, T_4=0.8794; p=0.4289; nicotine (preference) group, T_4=1.011; p=0.3692). Comparison of the mice with the same genotype of each group did not detect significant differences in the gene expression of *tbr1* compared to each other (Kruskal-Wallis test, wild-type mice, Kruskal-Wallis statistic = 5.385; p=0.9104; CB1$^{f/f;D1\text{-}Cre}$ animals Kruskal-Wallis statistic = 2.179; p=0.5360). Analysis with two way ANOVA did not find a significant effect for genotype ($F_{1,16}$=1.43; p=0.2485) or treatment ($F_{3,16}$=0.70; p=0.5645).

Analyses of the ratio of ΔCT for *egr2* (Fig. 80B) solely detected a significant difference in the group of naïve, untreated animals. CB1$^{f/f;D1\text{-}Cre}$ animals had a significant decrease in the *egr2* gene expression compared to their wild-type littermates (T_4=4.266; p=0.0130), implicating a basal difference in these animals as already seen for the candidate gene *klf4*. Further analyses for the gene regulation in the other groups could not find any additional, significant change in gene expression (saline group, WT vs. CB1$^{f/f;D1\text{-}Cre}$ mice T_4=0.9892; p=0.3786; nicotine (aversion) group WT vs. CB1$^{f/f;D1\text{-}Cre}$ animals T_4=0.1706; p=0.8728; nicotine (preference) group, WT vs. CB1$^{f/f;D1\text{-}Cre}$ mice T_4=1.023; p=0.3641). One way ANOVA with the animals of the same genotype of each group could not find significant differences in the gene expression of *egr2* (wild-type mice, F= 1.403; p=0.3111; CB1$^{f/f;D1\text{-}Cre}$ animals, F= 2.344; p=0.1492). Comparison of all groups with two way ANOVA did not detect a significant effect for treatment ($F_{3,16}$=1.22; p=0.3346).

The results for the relative gene expression changes of the last two candidate genes *neurod6* and *calml4* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ mice in different states of treatment are visualized in Fig. 81. No significant differences in the ratio of ΔCT could be found neither for the candidate gene *neurod6* (Fig. 81A) nor for *calml4* (Fig. 81B). By going more into detail and comparing the mice with the same genotype of each group, I could not reveal significant differences in the gene expression of *neurod6* (Kruskal-Wallis test, wild-type mice, Kruskal-Wallis statistic = 3.308; p=0.3466; CB1$^{f/f;D1\text{-}Cre}$ mice, Kruskal-Wallis statistic = 3.000; p=0.3916). By using two way ANOVA it was not possible to find any significant effect for genotype ($F_{1,16}$=1.04; p=0.3240), treatment ($F_{3,16}$=0.85; p=0.4859) or for the interaction of both genotype with treatment ($F_{3,16}$=0.84; p=0.4893) on the regulation of *neurod6*.

Same analyses were made for the candidate gene *calml4* (Fig. 81B). Also, one way ANOVA could not detect any significant differences in the ratio of ΔCT for this gene (one way ANOVA, wild-type mice, F=0.4250; p=0.7412; CB1$^{f/f;D1\text{-}Cre}$ mice, F=2.800; p=0.1086). A more stringent analysis with unpaired t test could not find any significant difference, but revealed a tendency for significance in the saline group (WT vs. CB1$^{f/f;D1\text{-}Cre}$ mice, T_4=2.275; p=0.0852). This effect could be seen as well for the comparison of untreated CB1$^{f/f;D1\text{-}Cre}$ mice with respective CB1$^{f/f;D1\text{-}Cre}$ animals, treated with vehicle (T_4=2.199; p=0.0928). Nonetheless, analy-

sis with two way ANOVA did not find a significant effect for genotype ($F_{1,16}$=0.08; p=0.7767), treatment ($F_{3,16}$=0.12; p=0.9497) or for the interaction of both factors ($F_{3,16}$=2.06; p=0.1486).

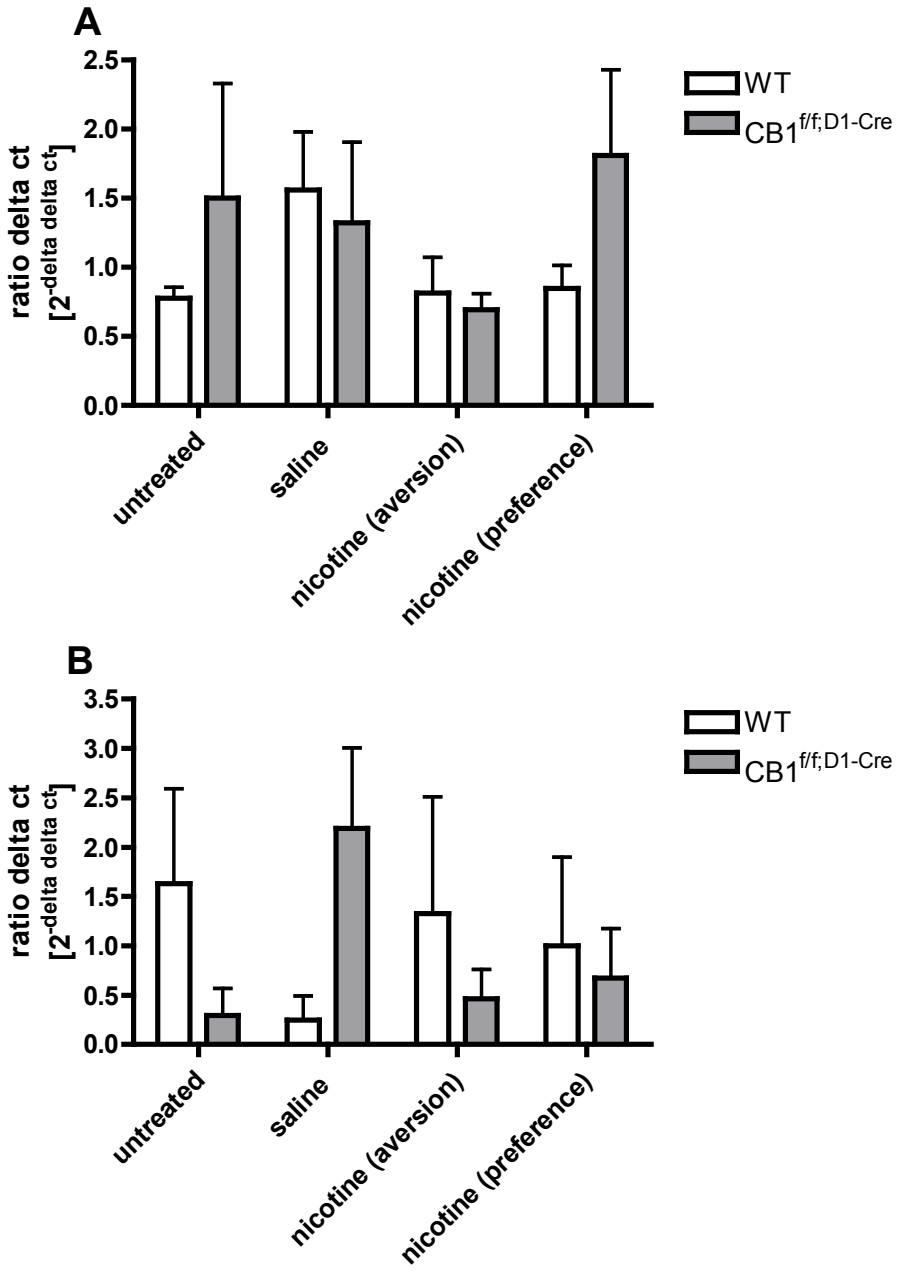

Fig. 81: Relative gene expression changes for the candidate genes *neurod6* (A) and *calml4* (B) in the striatum of CB1$^{f/f;D1\text{-}Cre}$ mice in different states of treatment. (A,B) No significant gene expression changes were neither for *neurod6* nor for *calml4* found in all groups. (n=3/group; male animals, 6-7 months old)

5.2.3. Additional gene expression analyses of candidate genes by quantitative real-time polymerase chain reaction in the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line

After the first gene expression analyses of the candidate genes, chosen from the GeneChip® Mouse Genome 430A 2.0 Array, I decided to repeat the quantitative real-time polymerase chain reaction (qPCR) experiment with the most promising genes *pttg1*, *klf4*, *egr2* and *calml4*. For control gene I used *cnr1* again. The decision for those genes was made because of their significant changes in the ratio of ΔCT in the basal state or after vehicle treatment of CB1$^{f/f;D1\text{-}Cre}$ mice. Repeated nicotine injections during a conditioned place preference (CPP) experiment seemed to have no influence on the alteration of those genes, but the stress of injection itself.

This second validation was made to test for possible differences between the CB1$^{f/f;D1\text{-}Cre}$ mouse line and the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line. Therefore I worked with brain tissue probes of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice after an equivalent CPP experiment, but used only the probes of vehicle-treated animals and additional untreated, naïve ones. The candidate genes were validated by qPCR by using TaqMan® MGB probes from Applied Biosystems. Results were again processed and analysed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) as already mentioned.

First, I measured by qPCR the control gene *cnr1*. The results are presented in Fig. 82. In J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice a significant decrease of *cnr1* expression in the striatum can be seen, compared to their respective wild-type independent from the same treatment. The ratio of ΔCT for the untreated J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice was 0.3084 ± 0.03886 compared to the ratio of ΔCT of 2.664 ± 0.2806 of untreated wild-type littermates. Student´s t test revealed that this difference in the ratio was very significant (T_3=8.316; p=0.0036). Vehicle treated littermates showed the same effect (T_3=6.668; p=0.0026). Two way ANOVA detected that these effects in both groups were not significant compared to each other for treatment ($F_{1,15}$=0.07; p=0.7990), only for the effect of genotype ($F_{1,15}$=115.17; p<0.0001).

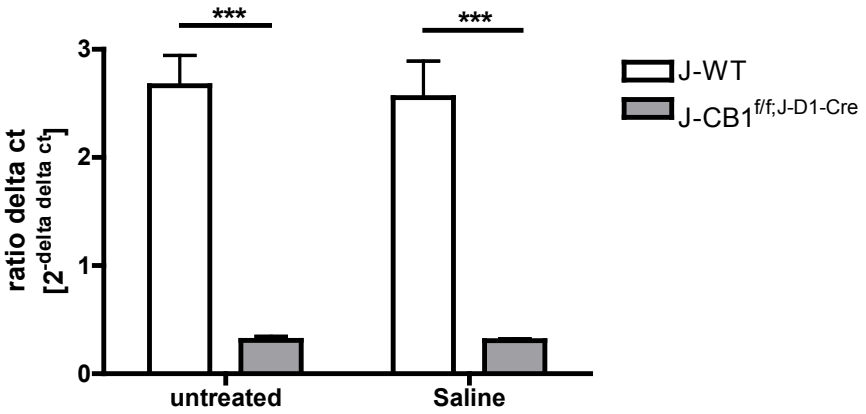

Fig. 82: Relative gene expression changes for the control gene *cnr1* in the striatum of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. Knock-out J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals in both groups display a significantly decreased gene expression compared to their respective wild-type (WT) littermates. ***p<0.001 (t test; n=4-5/group; male animals, 3-4.5 months old)

Next I evaluated the qPCR results for the two candidate genes *pttg1* (Fig. 83A) and *klf4* (Fig. 83B) in untreated and saline treated state. No significant differences in the ratio of ΔCT could be detected for the candidate gene *pttg1* (Fig. 83A). Two way ANOVA did not reveal a significant effect for genotype ($F_{1,15}$=0.60; p=0.4507), treatment ($F_{1,15}$=1.50; p=0.2420) or for the interaction of both factors ($F_{1,15}$=0.04; p=0.8398) on the regulation of this gene.

Fig. 83B presents the analyses of the ratio of ΔCT for the candidate gene *klf4*. I could not find a significant difference neither in the group of naïve, untreated animals nor in the saline treated group. The *klf4* gene expression in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ was not significantly altered. The ratio of ΔCT for the untreated J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice was 0.9188 ± 0.04991 compared to the ratio of ΔCT of 1.135 ± 0.1856 of untreated wild-type littermates. Unpaired t test with Welch´s correction revealed that this difference in the ratio was not significant (T_3=1.125; p=0.3425). Vehicle treated J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice showed a ratio of ΔCT of 1.452 ± 0.1331 compared to the ratio of ΔCT of 1.377 ± 0.07422 of saline treated wild-type littermates. This effect was also not significant (T_3=0.4934; p=0.6393). Comparisons of the mice of the same genotype of each group did not show differences in the gene expression of *klf4* for wild-type animals (T_7=1.319; p=0.2286), but a significant increase for J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice (T_8=3.752; p=0.0056).

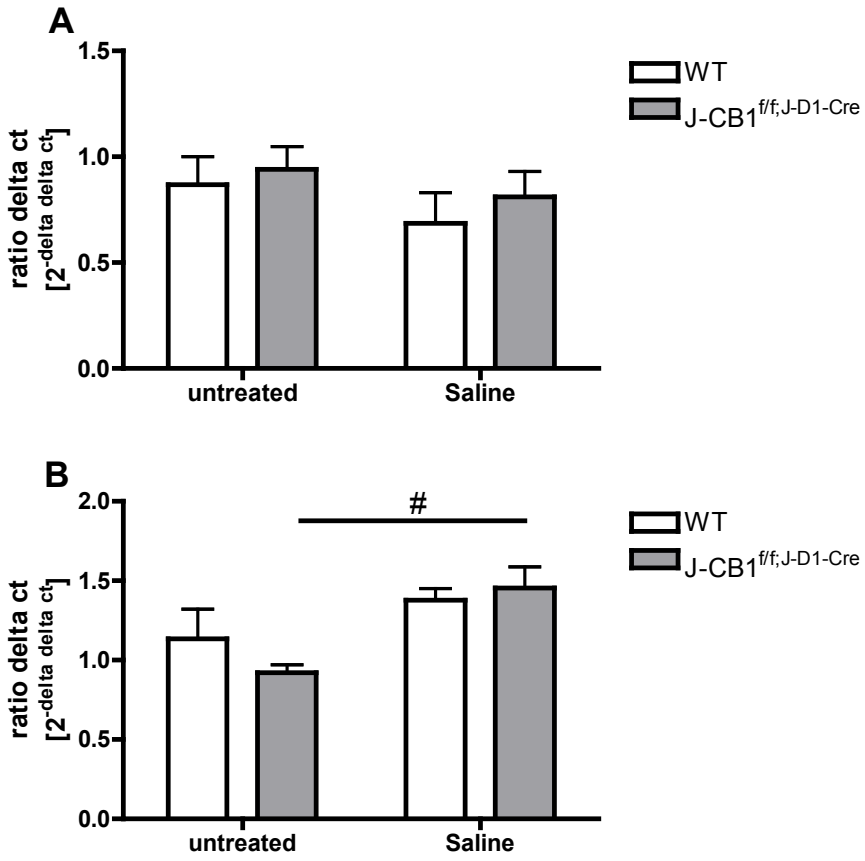

Fig. 83: Relative gene expression changes for the candidate genes *pttg1* (A) and *klf4* (B) in the striatum of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. (A) No significant differences in *pttg1* gene expression can be found in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals compared to their respective WT. (B) Knock-out J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals of the untreated group showed a significantly decreased gene expression of *klf4* compared to their respective saline treated knock-out littermates. $^{\#}p<0.05$ (significance between different treatment, same genotype) (t test; n=4-5/group; male animals, 3-4.5 months old)

The results for the relative gene expression changes of the other two candidate genes *egr2* (A) and *calml4* (B) in the striatum of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in both states of treatment are visualised in Fig. 84. Analyses of the ratio of ΔCT for *egr2* (A) could not find a significant difference in the group of naïve, untreated animals. J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals had no significant decrease in the *egr2* gene expression compared to their wild-type littermates (T_7=1.505; p=0.1761). Unpaired t test analyses for the gene regulation in the vehicle group could not find a significant change in gene expression neither (T_8=0.6077; p=0.5602). Comparisons of the mice of the same genotype of each group did not show differences in the gene expression of *egr2* for wild-type animals (T_7=1.060; p=0.3244), but a significant increase of gene expression for J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice (T_8=2.775; p=0.0241).

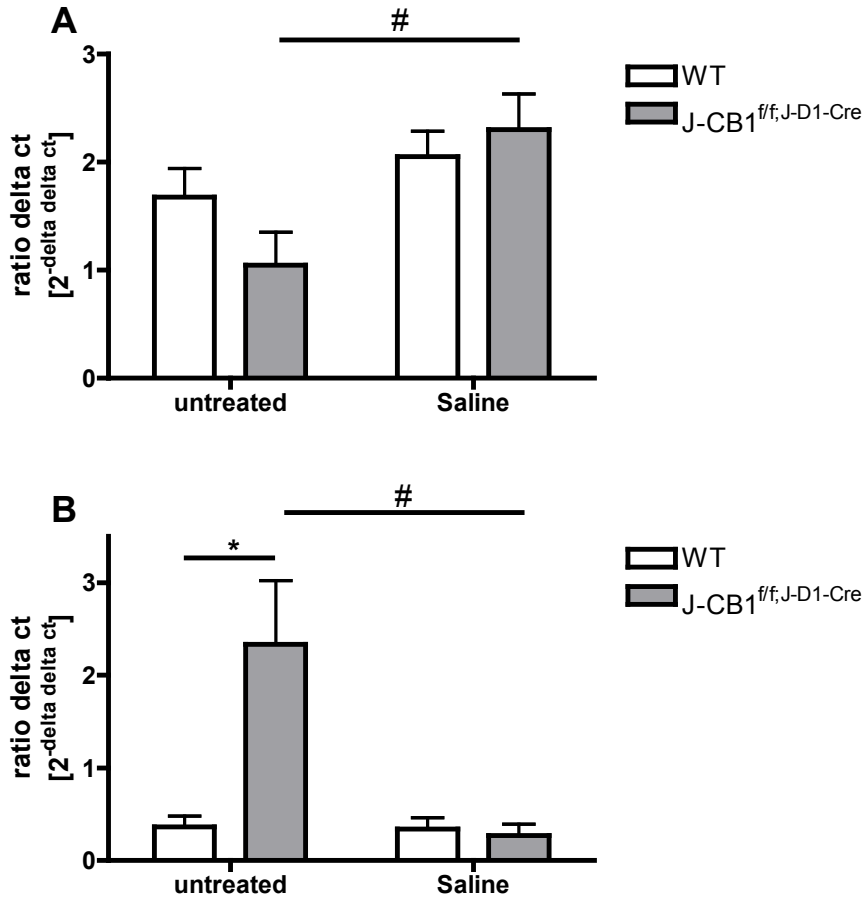

Fig. 84: Relative gene expression changes for the candidate genes *egr2* (A) and *calml4* (B) in the striatum of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. (A) No significant differences in *egr2* gene expression can be found in J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals compared to their respective WT. Knock-out J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals of the untreated group showed a significantly decreased gene expression of *egr2* compared to their respective saline treated knock-out littermates. (B) Knock-out J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals of the untreated group showed a significantly increased gene expression of *calml4* compared to their respective WT littermates. This increased gene expression is significantly decreased compared to their respective J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice of saline treated group. *p<0.05 (significance between different genotype, same treatment), $^{\#}$p<0.05 (significance between different treatment, same genotype) (t test; n=4-5/group; male animals, 3-4.5 months old)

Fig. 84B presents the analyses of the ratio of ΔCT for the candidate gene *calml4*. For this gene I could detect a significant difference in the group of naïve, untreated animals, but not in the saline treated group. The ratio of ΔCT for the untreated J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice was 2.335 ± 0.6858 compared to the ratio of ΔCT of 0.3615 ± 0.1173 of untreated wild-type littermates. Unpaired t test with Welch´s correction showed the significant difference of this effect (T_4=2.837; p=0.0470). Saline treated J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice showed a ratio of ΔCT of 0.2694 ± 0.1209 compared to the ratio of ΔCT of 0.3370 ± 0.1224 of saline treated wild-type littermates. This effect was not significant (T_8=0.3929; p=0.7046). Comparisons of the mice of the

same genotype of each group showed only a significant decrease in the gene expression of *calml4* for J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice (T_4=2.967; p=0.0413), but not for wild-type animals (T_7=0.1417; p=0.8913).

5.2.4 Comparison of gene expression analyses of CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse lines

After gene expression analyses of the candidate genes, chosen from the GeneChip® Mouse Genome 430A 2.0 Array, I compared the results gained from CB1$^{f/f;D1\text{-}Cre}$ mouse line with the results from J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line in order to find possible differences or analogues in gene expression.

Starting with the expression of the control gene *cnr1,* the results are presented in Fig. 85. As already mentioned before, both CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice generally showed a significantly lower *cnr1* expression compared to their respective wild-type littermates, independent how they were treated (untreated group: CB1$^{f/f;D1\text{-}Cre}$, T_4=4.295, p=0.0127; J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$, T_3=8.316; p=0.0036; vehicle treated animals: CB1$^{f/f;D1\text{-}Cre}$, T_4=3.320; p=0.0294; J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$, T_3=6.668; p=0.0026). Two way ANOVA followed by Bonferroni Post Hoc detected that these effects in both groups were highly significant compared to each other for genotype ($F_{3,23}$=62.80; p<0.0001), but not for the interaction of treatment and genotype ($F_{3,23}$=0.32; p=0.8100) and for the effect of treatment itself ($F_{1,23}$=0.58; p=0.4525). Additionally, Bonferroni post test showed a significantly higher gene expression ratio of *cnr1* for the wild-type mice of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line compared to the wild-type animals of CB1$^{f/f;D1\text{-}Cre}$ mouse line as well in the untreated group (T_5=8.037; p<0.001) as in the saline treated group (T_6=7.960; p<0.001). This effect could not be found in the knock-out animals of either group.

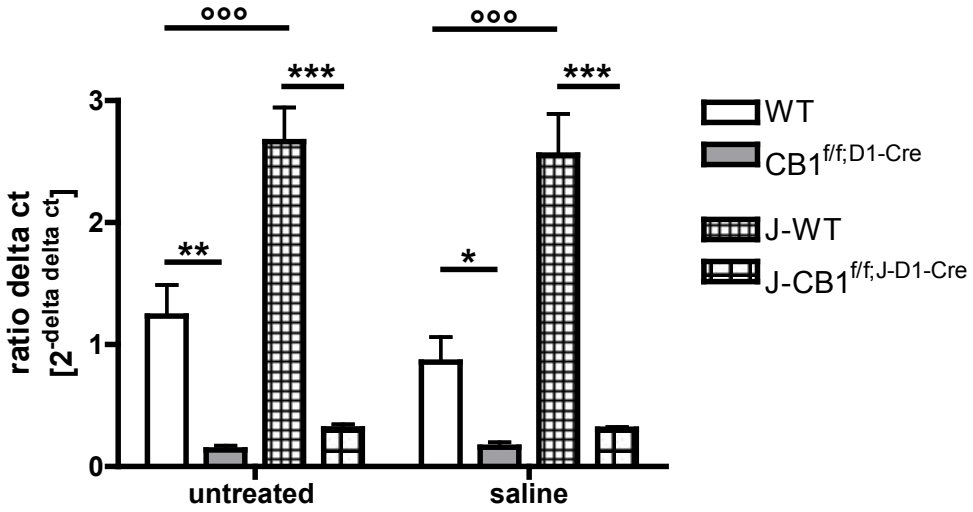

Fig. 85: Relative gene expression changes for the control gene *cnr1* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. Knock-out CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ animals in both groups display a significantly decreased gene expression compared to their respective wild-type (WT) littermates. *p<0.05, **p<0.01, ***p<0.001 (significance between different genotype, same genetic background, same treatment), °°°p<0.001 (significance between same treatment, same genotype, different genetic background (t test; J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$: n=4-5/group, male animals, 3-4.5 months old; CB1$^{f/f;D1\text{-}Cre}$: n=3/group, male animals, 6-7 months old)

The comparison of the gene expression of *pttg1* (Fig. 86) in both conditional mouse lines detected only significant differences in the group of vehicle treated animals. All untreated mice showed the similar gene expression of this candidate gene. Saline treated CB1$^{f/f;D1\text{-}Cre}$ mice differed significantly from their wild-type littermates and showed a decrease of *pttg1* expression (T_6=4.655; p<0.001). Post hoc analyses revealed no significant change in gene expression for J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice compared to their respective wild-type animals, but could show that there is a significant difference in gene expression both for the knock-out mice and the wild-type mice of both mouse lines. Wild-type animals of CB1$^{f/f;D1\text{-}Cre}$ mouse line showed a significantly higher expression of *pttg1* than their respective wild-type animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line (T_6=2.731; p<0.05). Two way ANOVA could find a significant genotype effect ($F_{3,23}$=5.48; p=0.0054), but no significance for the effect of treatment ($F_{1,23}$=1.12; p=0.3011). Additionally, Bonferroni post test could find a significant gene expression decrease for the knock-out mice of the CB1$^{f/f;D1\text{-}Cre}$ line compared to the respective knock-out animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line (T_7=2.994; p<0.05).

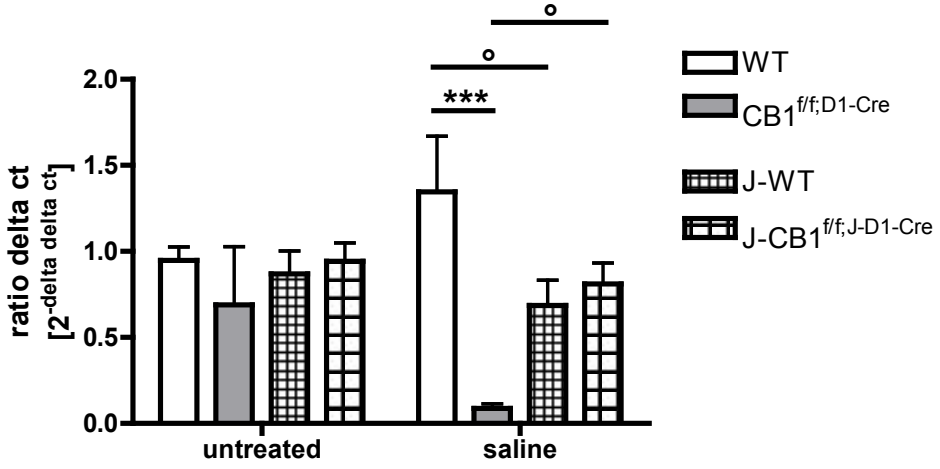

Fig. 86: Relative gene expression changes for the candidate gene *pttg1* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. Saline treated knock-out CB1$^{f/f;D1\text{-}Cre}$ mice show a significantly decreased gene expression compared to their respective WT. J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice with saline treatment displayed a significantly increased gene expression compared to the knock-out animals with a different background. For the WT an opposite effect (J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$: significantly lower than CB1$^{f/f;J\text{-}D1\text{-}Cre}$) was seen. ***p<0.001 (significance between different genotype, same background, same treatment), °p<0.05 (significance between same treatment, same genotype, different genetic background (t test; J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$: n=4-5/group, male animals, 3-4.5 months old; CB1$^{f/f;D1\text{-}Cre}$: n=3/group, male animals, 6-7 months old)

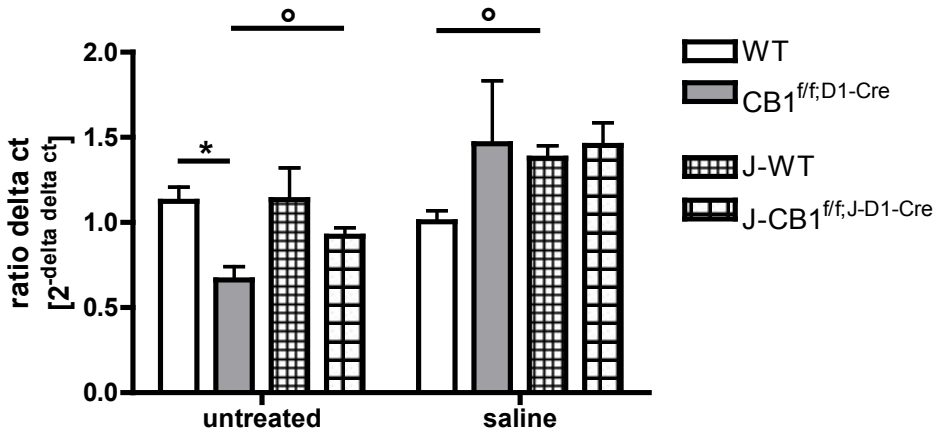

Fig. 87: Relative gene expression changes for the candidate gene *klf4* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. Untreated knock-out CB1$^{f/f;D1\text{-}Cre}$ mice show a significantly decreased gene expression compared to their respective WT. J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice of the same group displayed a significantly increased gene expression compared to the knock-out animals with a different background. Saline treated wild-type animals on the J-background showed a significantly increased gene expression compared to the wild-type mice of the other background. *p<0.05 (significance between different genotype, same background, same treatment), °p<0.05 (significance between same treatment, same genotype, different genetic background (t test; J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$: n=4-5/group, male animals, 3-4.5 months old; CB1$^{f/f;D1\text{-}Cre}$: n=3/group, male animals, 6-7 months old)

Fig. 87 shows the relative gene expression changes for the candidate gene *klf4* in the striatum CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. Only the gene expression of untreated knock-out mice of CB1$^{f/f;D1\text{-}Cre}$ mouse line is significantly decreased compared to their wild-type littermates (T_4=4.008; p=0.0160). Using two way ANOVA, I could find a significant treatment effect ($F_{3,23}$=11.99; p=0.0021), but not for the effect of genotype ($F_{1,23}$=0.83; p=0.4902)). Yet, I found significance for the interaction of treatment and genotype ($F_{3,23}$=3.05; p=0.0489). Post hoc analyses revealed that untreated knock-out animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line have a significantly increased *klf4* expression than the untreated knock-out animals of the CB1$^{f/f;D1\text{-}Cre}$ mouse line (T_6=2.919; p=0.0267). A similar increase of *klf4* was seen for vehicle treated wild-type animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line compared to vehicle treated wild-type mice of CB1$^{f/f;D1\text{-}Cre}$ line (T_6=3.396; p=0.0146).

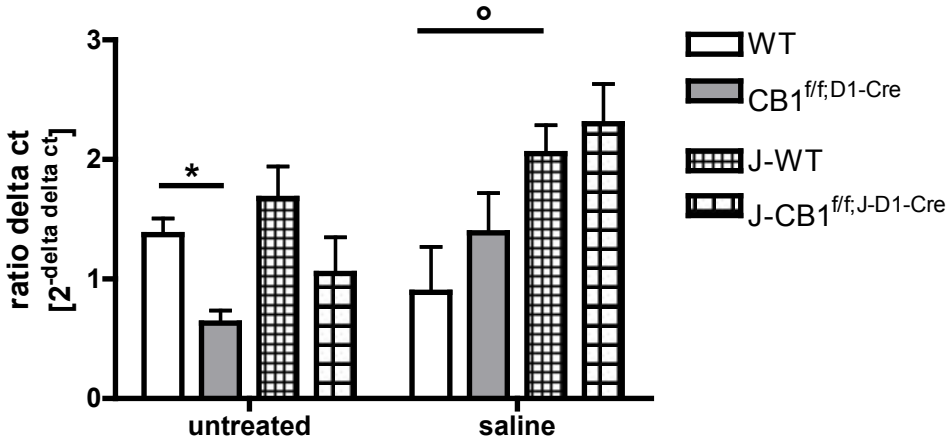

Fig. 88: Relative gene expression changes for the candidate gene *egr2* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. Untreated knock-out CB1$^{f/f;D1\text{-}Cre}$ mice show a significantly decreased gene expression compared to their respective WT. Saline treated wild-type animals on the J- background showed a significantly increased gene expression of *egr2* compared to wild-type mice of the N- background. *p<0.05 (significance between different genotype, same background, same treatment), °p<0.05 (significance between same treatment, same genotype, different genetic background (t test; J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$: n=4-5/group, male animals, 3-4.5 months old; CB1$^{f/f;D1\text{-}Cre}$: n=3/group, male animals, 6-7 months old)

As it can be seen in Fig. 88, the comparison of the gene expression of *egr2* in both conditional mouse lines found only a significant difference in the group of untreated animals. Untreated CB1$^{f/f;D1\text{-}Cre}$ mice had a significantly decrease of *egr2* expression compared to their respective wild-type littermates (T_4=4.266; p=0.0130). Two way ANOVA found a significant genotype ($F_{3,23}$=3.70; p=0.0263) and a significant treatment effect ($F_{1,23}$=4.94; p=0.364), but no significant interaction of both factors ($F_{3,23}$=2.93; p=0.0552). Post hoc analyses showed a

significant gene expression increase for the wild-type mice of the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ line compared to the respective wild-type animals of CB1$^{f/f;D1\text{-}Cre}$ mouse line (T_6=2.774; p=0.0322).

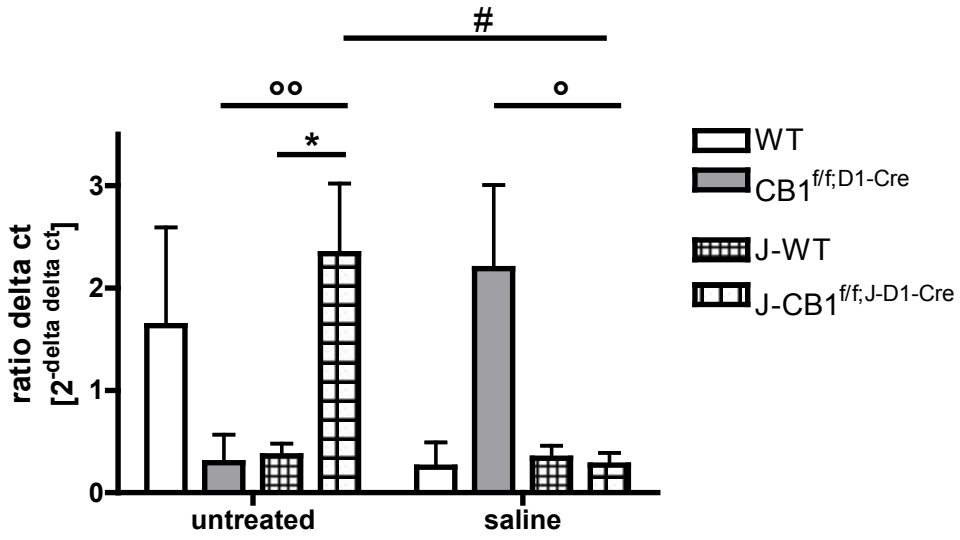

Fig. 89: Relative gene expression changes for the candidate gene *calml4* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice in different states of treatment. Untreated knock-out J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice show a significantly increased gene expression compared to their respective WT. No additional differences in gene expression for the other groups could be found. *p<0.05 (significance between different genotype, same genetic background, same treatment), #p<0.05 (significance between same genotype, same genetic background, different treatment) (t test; J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$: n=4-5/group, male animals, 3-4.5 months old; CB1$^{f/f;D1\text{-}Cre}$: n=3/group, male animals, 6-7 months old)

The comparison of the gene expression of the last candidate gene *calml4* (Fig. 89) found a significant increase of the gene in the group of untreated animals. Untreated J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice express significantly more *calml4* compared to their wild-type littermates (T_4=2.837; p=0.0470). Additionally, compared to their respective saline treated knock-out littermates a decrease of *calml4* expression can be detected after vehicle treatment (T_4=2.967; p=0.0413). Two way ANOVA could find a significance for the interaction of treatment and genotype ($F_{3,22}$=7.05; p=0.0017), but no significant genotype ($F_{3,22}$=2.16; p=0.1211) and no significant treatment effect ($F_{1,22}$=1.39; p=0.2515). Bonferroni post test found a significant gene expression decrease for untreated knock-out mice of the CB1$^{f/f;D1\text{-}Cre}$ line as compared to the respective untreated knock-out animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line (T_6=3.199; p<0.01). In addition, a significant gene expression increase of *calml4* in saline treated knock-out mice of the CB1$^{f/f;D1\text{-}Cre}$ line compared to the respective vehicle treated knock-out animals of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line (T_6=3.007; p<0.05).

5.3. Behavioural analyses with NG2-EYFP mouse line

5.3.1. Characterization of basic functions

5.3.1.1 Measurement of body weight

Before I started with the actual series of behavioural experiments, I measured the weight of the animals. As seen in Fig 90, NG2-EYFP mice do not differ from their respective wild-type littermates (T_{40}=1.521, p=0.1362). Naïve NG2-EYFP mice weighted 30.50 ± 0.9778 g whereas their wild-type littermates had 32.68 ± 0.7692 g.

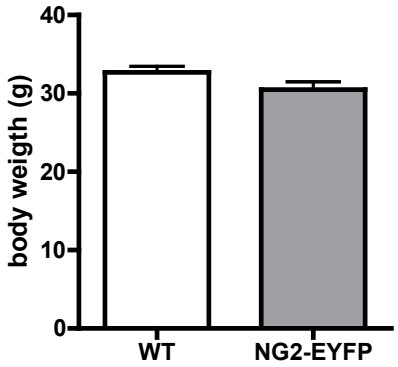

Fig. 90: Body weight of naïve NG2-EYFP and WT animals. No significant differences were observed between the groups (t test; n=27/ NG2-EYFP and 15/WT; male animals; 4-8 months old).

5.3.1.2 Acoustic startle response

Deficits in the auditory system can change the behaviour of animals in special experiments. In order to know whether the mice have a decrease or loss in hearing, the animals were tested in ASR. Fig. 91 shows that NG2-EYFP mice respond with a significant lower reaction to loud noise than the wild-type animals (T_{40}=2.634, p=0.0119).

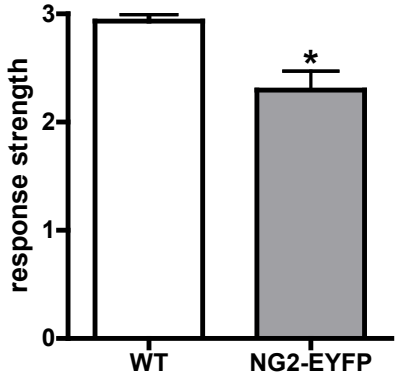

Fig. 91: Response strength of NG2-EYFP and WT animals. Significant differences in reaction to sound stimulus were observed between the groups, *p<0.05 (t test; n=27/ NG2-EYFP and 15/WT; male animals; 4-8 months old).

5.3.2. Models of locomotion and motor functions

5.3.2.1 Open Field test

In the first experiment I aimed at analysing the basal locomotion of naïve wild-type and NG2-EYFP mice in an open field box for 10 min. The wild-type animals moved a total distance of 3602 ± 143.8 cm, while the NG2-EYFP knock-out mice moved a total distance of 3891 ± 210.8 cm. As shown in Fig. 92, no significant difference in the distance moved (T_{45}=1.154, p=0.2545) and the time spent in centre (T_{45}=0.4973, p=0.6214) was detected between genotypes.

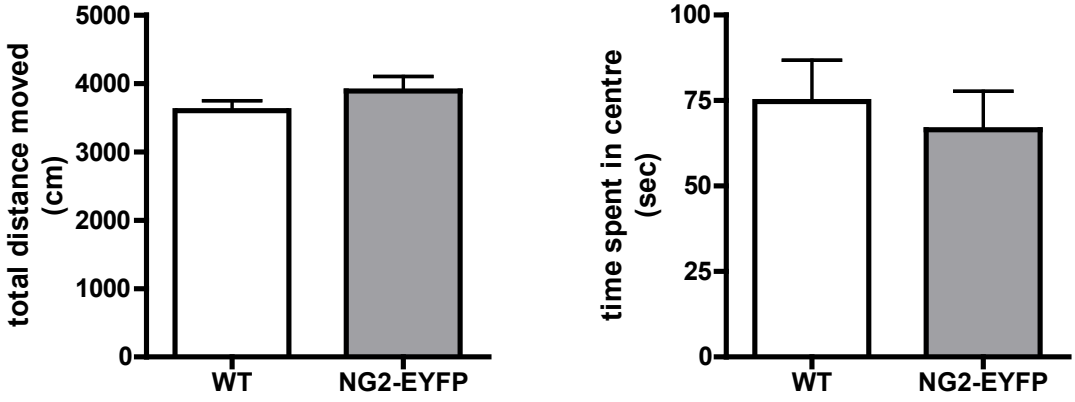

Fig. 92: Basal levels in locomotion and time of NG2-EYFP and wild-type littermate controls (WT). No significant difference in distance moved and time spent in centre was detected (t test; n=25/ WT and n=22/NG2-EYFP; male animals, 4-6 months old).

5.3.2.2 Grip strength test

Next I tested the grip strength of the animals comparing batches of different ages. In Fig. 93, the basal level of WT and NG2-EYFP mice is shown. Mature wild-type animals showed grip strength of 100.1 ± 4.042 p, while their NG2-EYFP littermates showed grip strength of 100.2 ± 4.022 p. Therefore, mutant mice showed no significant difference in grip strength (T_{29}=0.005196, p=0.9959). Additionally, old animals were tested in this paradigm. Old wild-type animals displayed grip strength of 68.28 ± 5.392 p, while their NG2-EYFP littermates showed grip strength of 58.89 ± 2.158 p. No significant differences could be seen in the group of old animals (T_{10}=1.616, p=0.1371).

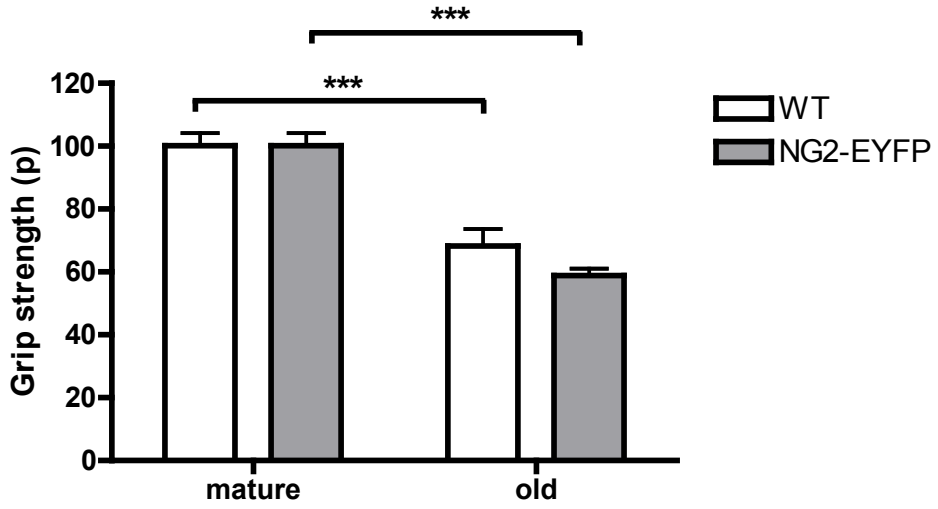

Fig. 93: Measurement of grip strength in NG2-EYFP. Age decreased grip strength in WT animals with the similar efficacy in NG2-EYFP. ***p<0.0001 (2-WAY ANOVA followed by Bonferroni test; n=15-16/ mature and n=6/ old group; male animals, old animals: 19 months, mature animals: 4-6 months)

Compared to younger mice of the same genotype, old NG2-EYFP mice and their wild-type littermates revealed a very significant loss of strength ($F_{1,39}$=54.12; p<0.0001). In this experiment, no significant interaction between the genotype and age ($F_{1,39}$=0.90; p=0.3491) was found. Furthermore there was no significant result for genotype effects ($F_{1,39}$=0.89; p=0.3521).

5.3.2.3 Rotarod test

NG2-EYFP mice and their wild-type littermates were tested in differences in performance and time on the rotarod. These differences are shown in Fig. 94. Both, NG2-EYFP animals (T_4=4.907, p=0.0080) and WT mice (T_4=3.676, p=0.0213) showed a significant increase in time on the rod between session 1 and 3.

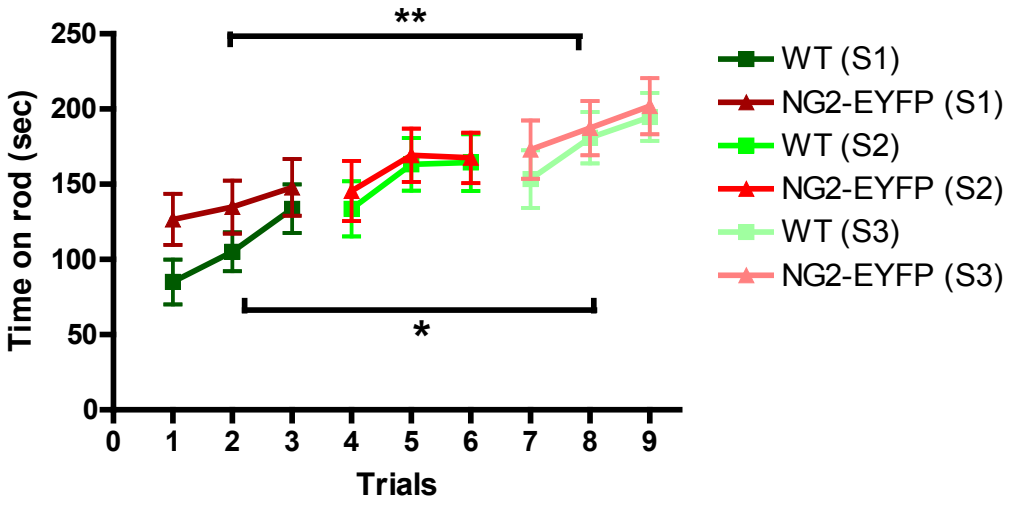

Fig. 94: Time spent walking on the rod of NG2-EYFP mice compared to their WT littermates. Both groups increased significantly their time spent walking over repeated sessions.**p<0.01 for NG2-EYFP mice and *p<0.05 for wild-type littermates (t test; n=20/ NG2-EYFP and 18/WT; male animals, 8-12 months old)

5.3.3 Models of emotion

5.3.3.1 Elevated plus maze test

I investigated the behaviour of the mice in the models of emotion. In the EPM the basal anxiety differences between both genotypes could be revealed (Fig. 95). No differences in the percentage of time spent in open arms (T_{47}=0.6778, p=0.5013) and percentage of entries in open arms (T_{47}=1.618, p=0.1123) were detected. Wild-type animals spent 4.057 ± 1.879% of total time in the open arms, which constituted 12.70 ± 2.089% of the entries. The NG2-EYFP mice spent 5.516 ± 0.9965% of total time in the open arms, which constituted 18.00 ± 2.544% of the entries.

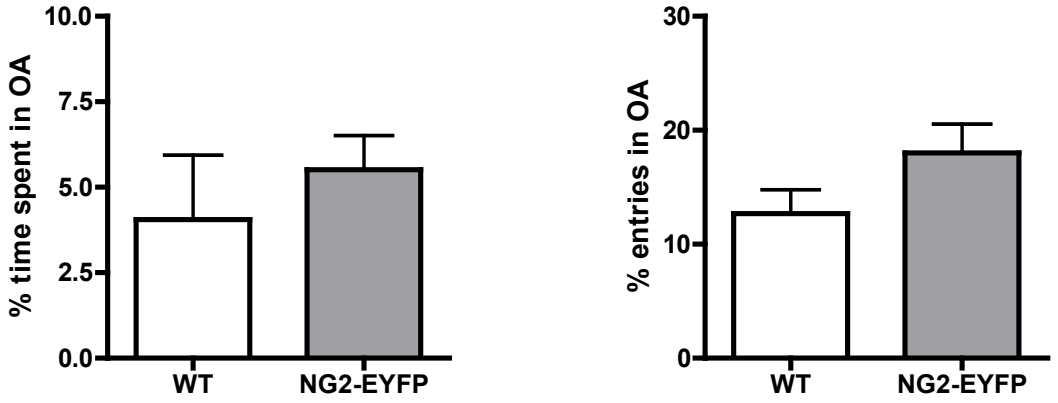

Fig. 95: Percentage of time and entries of NG2-EYFP and WT animals in open arms of the EPM. No difference was detected (t test; n=24/ NG2-EYFP and 25/WT; male animals, 5.5-8 months old).

Fig. 96 shows the behaviour of the animals pertaining to risk assessment and rearings into the open arms, two special parameters measurable during EPM. There were no significant differences found for risk assessment (T_{29}=1.880, p=0.0701) and number of rearings into open arms (T_{29}=0.3788, p=0.7076).

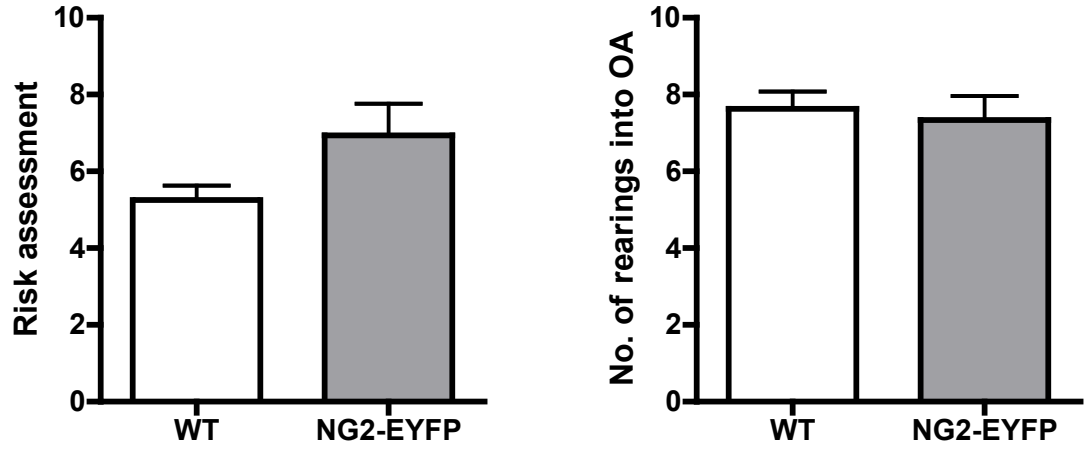

Fig. 96: Basal levels in risk assessment and number of rearings into open arms of NG2-EYFP and WT. No difference was detected (t test; n=15/ NG2-EYFP and 16/WT; male animals, 5.5-7.5 months old).

5.3.3.2 Light-dark test

The light-dark-test (LD) takes advantage of the natural conflict between the tendency to explore unknown areas and the aversion of a brightly lit open environment. With this test I measured the activity of the animals in exploration. In Fig. 97, the basal behaviour of WT and

NG2-EYFP is shown. The results of time spent in the lit compartment (T_{46}=2.391, p=0.0210) and entries into the lit compartment (T_{46}=1.921, p=0.0610) revealed significant genotype-related differences for the time, but only a tendency for the entries. The naïve wild-type mice spent 27.67 ± 3.117 sec in the lit compartment and showed 3.917 ± 0.4461 entries in lit compartment. At the same time, the NG2-EYFP animals spent 40.00 ± 4.111 sec and made 5.125 ± 0.4436 entries in the lit compartment.

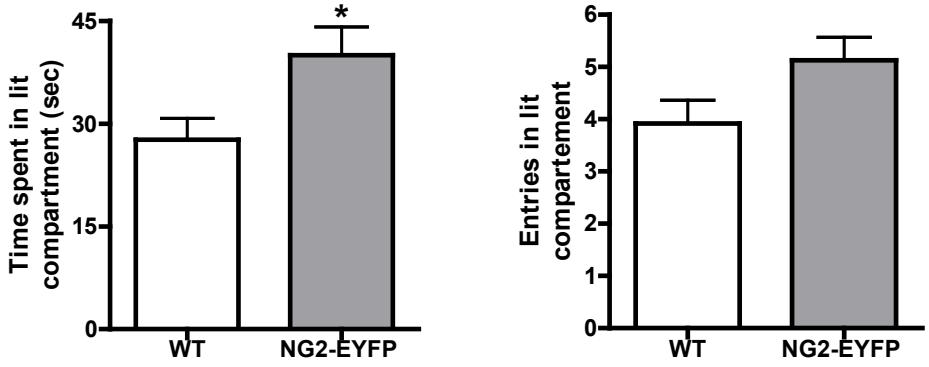

Fig. 97: Time spent and entries of NG2-EYFP and WT mice in the lit compartment in the light-dark test. Significant differences for the time spent in lit compartment were detected. *p<0.05 compared to the respective WT animals (t test; n=24/ NG2-EYFP and 24/WT; male animals, 4-8 months old).

5.3.3.3 Forced Swim test

In Fig. 98, the basal level of floating time in NG2-EYFP and WT animals is shown. In this experiment, a strong significant difference between the groups was found (T_{40}=3.545, p=0.0010). NG2-EYFP mice spent only 113.8 ± 11.28 sec in floating, while their respective wild-type littermates float 170.4 ± 6.469 sec.

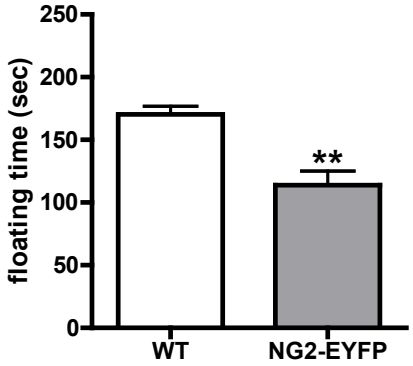

Fig. 98: Floating time in NG2-EYFP and WT animals. Significant decrease in time spent floating was detected in NG2-EYFP. **p<0.001 compared to the respective WT (t test; n=27/ NG2-EYFP and 15/WT; male animals; 5-13 months old).

5.3.4 Models of recognition memory and learning

5.3.4.1 Novel object recognition task

The time spent in exploration during training period is shown in Fig. 99. Neither genotype showed differences in exploration or a natural preference for one of the objects. No significant interaction between the genotype and objects could be observed ($F_{1,82}$=0.15; p=0.6984). Additionally, no further significance is revealed, either in the effect of genotype ($F_{1,82}$=1.82; p=0.1814) or in the effect of objects ($F_{1,82}$=0.03; p=0.8618).

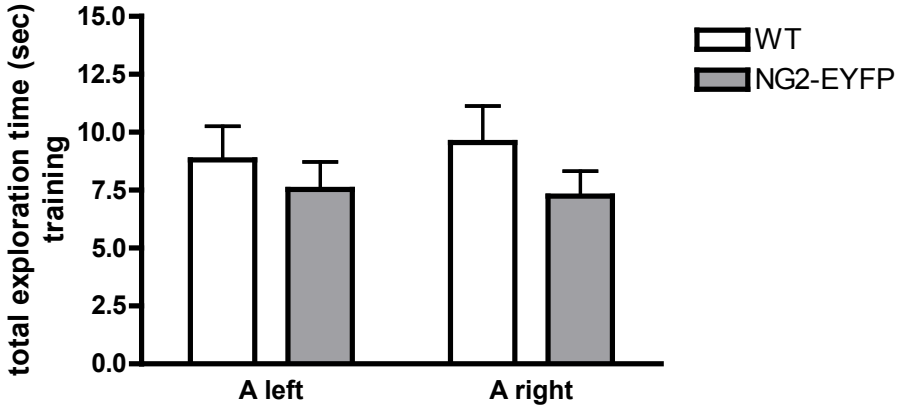

Fig. 99: Total time spent in exploration of object A in NG2-EYFP compared to WT animals. No significant differences could be seen between genotypes. (2-WAY ANOVA followed by Bonferroni test; n=27/ NG2-EYFP and 16/WT; male animals, 4-6 months old).

After two hours of retention a significant effect in exploration of the new object is shown in Fig. 100 ($F_{1,82}$=5.27; p=0.0243) for both groups. However, I did not observe genotype-related differences ($F_{1,82}$=0.09; p=0.7598).

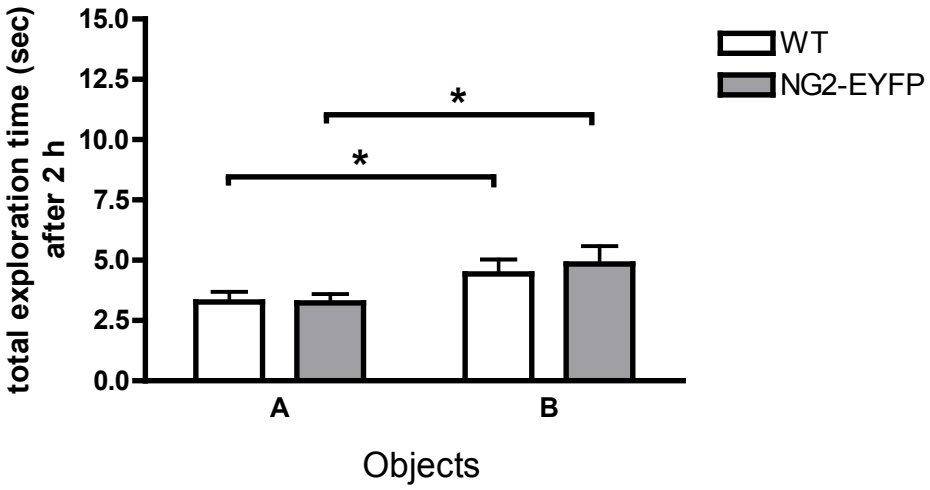

Fig. 100: Total time spent on exploration after two hours of retention in NG2-EYFP compared to WT animals in the NORT. Object B was significantly more explored in both groups. *p<0.05 compared to object A (2-WAY ANOVA followed by Bonferroni test; n=27/ NG2-EYFP and 16/WT; male animals, 4-6 months old).

Fig. 101 represented the same effects as seen in Fig. 100 after 24 hours of retention. Both genotypes showed a significant increase of exploration of object C ($F_{1,82}$=17.14; p<0.0001), but no significant effect was seen for the genotype ($F_{1,82}$=0.15; p=0.6962).

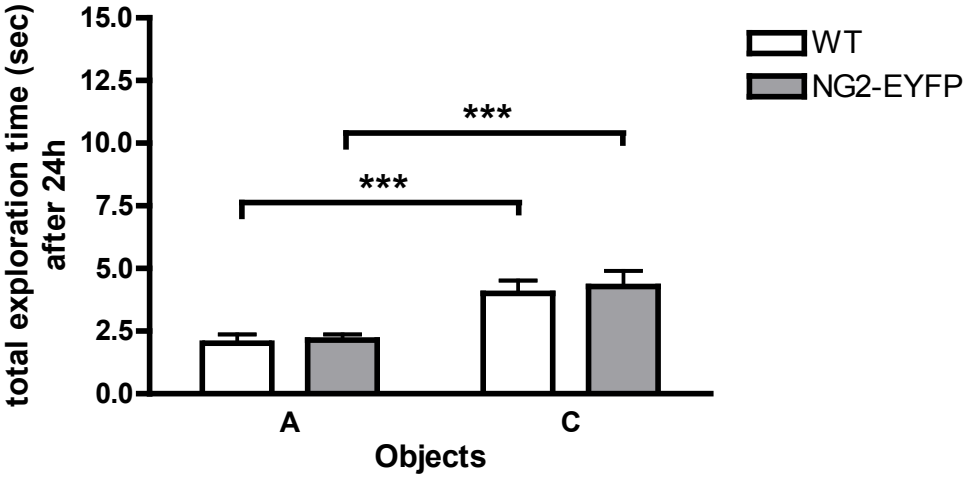

Fig. 101: Total time spent on exploration after 24 hours of retention in NG2-EYFP compared to WT littermates in the NORT. Object C was significantly more explored in both groups. ***p<0.0001 compared to object A (2-WAY ANOVA followed by Bonferroni test; n=27/ NG2-EYFP and 16/WT; male animals, 4-6 months old).

5.3.4.2 Morris water maze task

Presented in Fig. 102 it can be seen that NG2-EYFP and their WT littermates significantly decrease path length over all trials ($F_{4,90}$=15.96; p<0.0001). However, I could not detect genotype-related differences in the path length ($F_{1,90}$=0.20; p=0.6585). On first day NG2-EYFP significantly start with a shorter pathway compared to WT animals (T_{18}=2.847, p=0.0107)

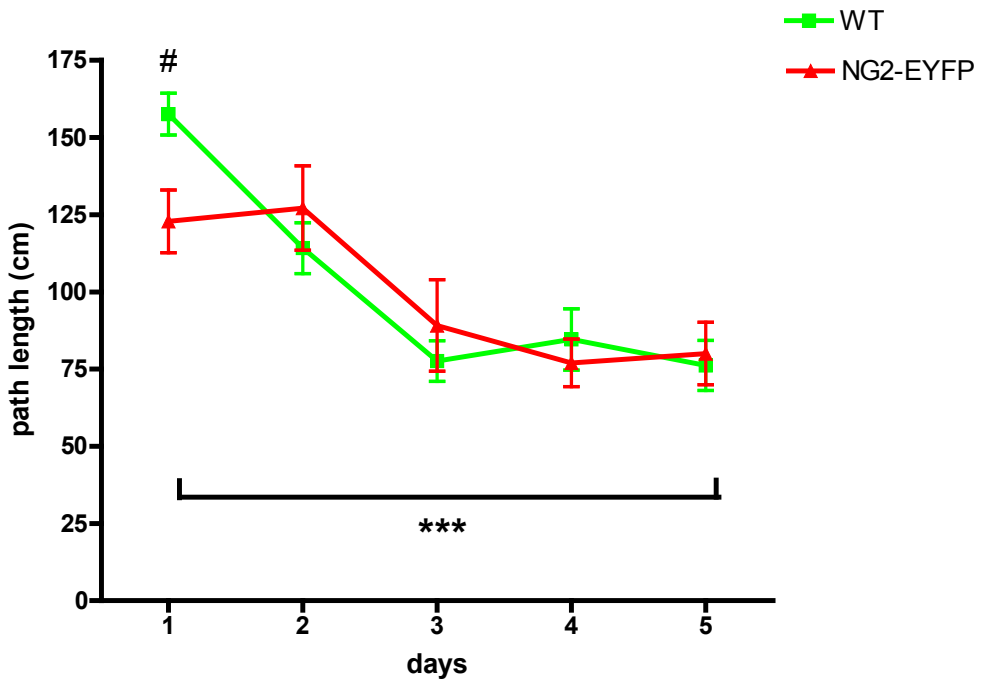

Fig. 102: Total path length swum to escape the water over all days for NG2-EYFP compared to WT littermates. WT animals start with a significantly longer path way than NG2-EYFP mice, while both genotypes significantly reduce the distance swum over the course of the experiment. #p<0.05 compared to NG2-EYFP mice, ***p<0.0001 (2-WAY ANOVA followed by Bonferroni test; n=10/ group; male animals, 6.5-8.5 months old).

Fig. 103 reveals no significant differences in escape latency between NG2-EYFP and respective WT littermates ($F_{4,90}$=1.95; p=0.1089) for all experimental days. Additionally, no genotype-related difference ($F_{1,90}$=0.01; p=0.9368) can be seen.

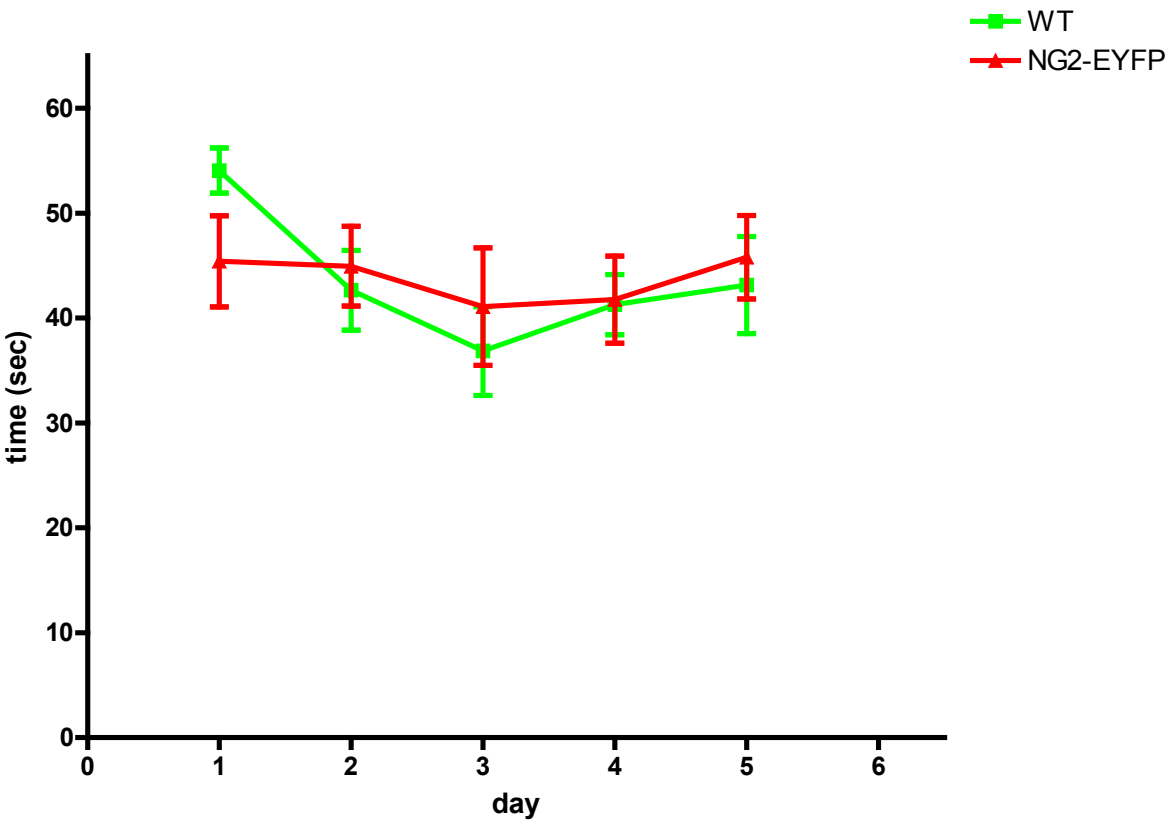

Fig. 103: Escape latency to platform of NG2-EYFP mice compared to respective WT littermates. No significant differences during the experiment were observed (2-WAY ANOVA followed by Bonferroni test; n=10/ group; male animals, 6.5-8.5 months old).

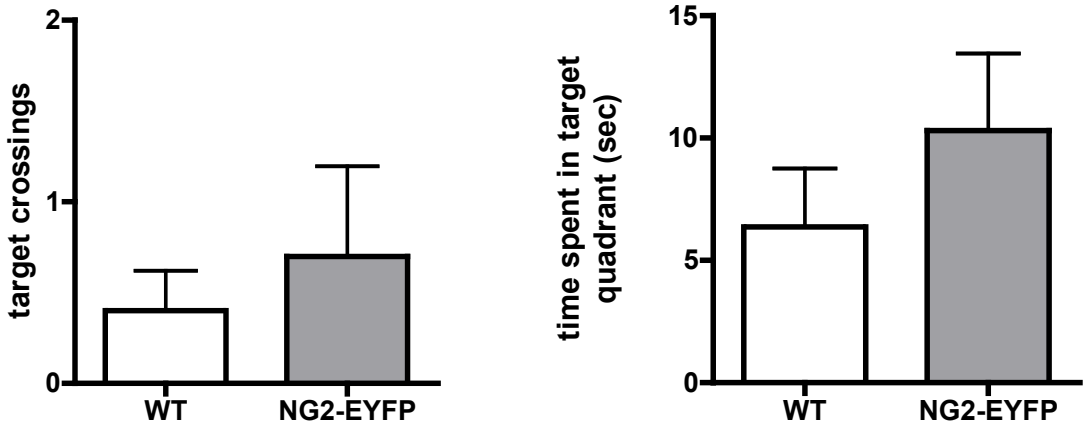

Fig. 104: Basal levels of target crossing and time spent in target quadrant of NG2-EYFP and WT mice during probe trial. No significant differences for target crossings and time spent in target quadrant were detected. (t test; n=10/ group; male animals, 6.5-8.5 months old).

In Fig. 104, the behaviour of WT and NG2-EYFP mice during probe trial is shown. The results of target crossings (T_{18}=0.5529, p=0.5872) and time spent in target quadrant (T_{18}=0.9995, p=0.3308) present no differences between the two groups. The wild-type mice crossed the target 0.4000 ± 0.2211 times and spent 6.370 ± 2.381 sec in the target quadrant. NG2-EYFP animals crossed the target 0.7000 ± 0.4955 times and spent 10.31 ± 3.142 sec in the target quadrant.

5.3.5 Model of excitotoxic seizures

Fig. 105 shows the behavioural effects in NG2-EYFP and C57BL/6N wild-type animals after injection of kainic acid at dose of 30 mg/kg. The response strength of NG2-EYFP mice is significantly reduced over the whole scoring time (Mann-Whitney U=9.500, p=0.0148).

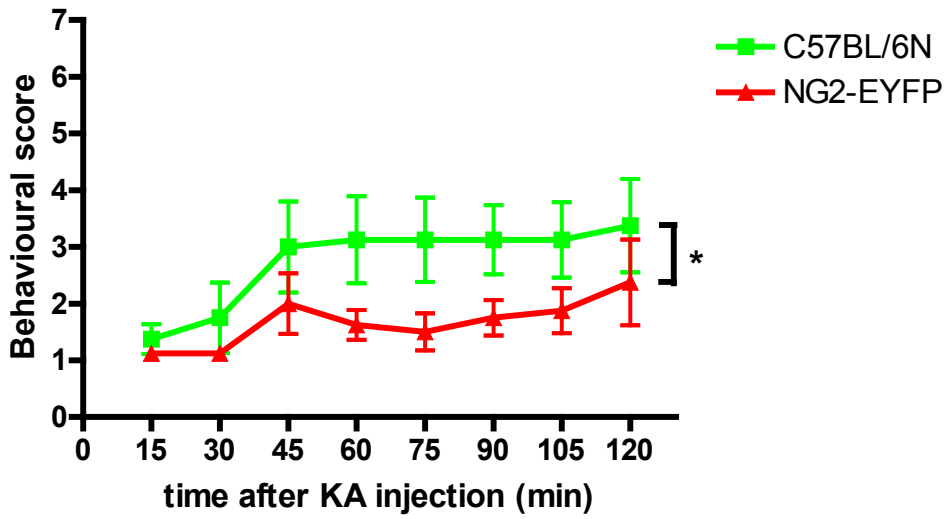

Fig. 105: Behavioural effects in NG2-EYFP mice compared to age-matched C57BL/6N animals after injection of kainic acid (30 mg/kg, i.p.). Seizure severity was significantly reduced in NG2-EYFP mice at every time point of scoring compared to the WT controls. *p<0.05 (Mann Whitney test, two-tailed; n=6/ NG2-EYFP and 6/C57BL/6N; male animals, 9-10 months old and n=2/ NG2-EYFP and 2/WT; male animals, 24-25 months old).

6. Discussion

6.1 CB1 receptor conditional mutant mice and the endocannabinoid system

The aim of this study was to show how the CB1 receptor in D1 dopamine receptor expressing neurons is involved in the regulation of different behaviour, such as locomotion, anxiety, and addiction. To this end, I used the conditional mutant mouse line $CB1^{f/f;D1\text{-}Cre}$ in order to explore its responses in different models of behaviour in order to reveal whether the pathways, affected by the Cre recombinase-mediated deletion of CB1 receptor in D1 receptor expressing neurons, play any role in a distinct behaviour. The conditional mutant mouse line $CB1^{f/f;D1\text{-}Cre}$ was generated by crossing of $CB1^{f/f}$ mice with the transgenic mouse line expressing Cre recombinase under the control of the regulatory sequences of the dopamine receptor D1, in order to obtain a deletion of CB1 receptor in striatal GABAergic neurons (Lemberger et al., 2007; Mantamadiotis et al., 2002).

Double *in situ* hybridization with CB1 and D2 dopamine receptor riboprobes showed that the CB1 receptor is absent in the great majority of non-D2 expressing neurons of the conditional mutant mice, indicating the general deletion of the CB1 receptor gene in neurons expressing the D1 receptor (Monory et al., 2007), which is consistent with the expected recombination pattern induced by the Cre recombinase under the control of D1 regulatory sequences (Mantamadiotis et al., 2002). All other non-D1 and non-D2 neurons in the striatum keep their expression of CB1 receptor in the mutant mice. These neurons very likely belong to the population of striatal GABAergic interneurons, which were shown to contain CB1 mRNA (Hohmann and Herkenham, 2000). However, in agreement with the expected recombination pattern of D1-Cre mice (Lemberger et al., 2007), a certain number of D2-expressing neurons appear to be also affected by Cre-mediated recombination, as the number of double CB1/D2-expressing neurons was decreased in mutant mice (Monory et al., 2007). Other studies reported that the over-lap between D1- and D2-expressing neurons in the striatum might be higher than generally proposed (Aizman et al., 2000). It was suggested that CB1 receptor signalling is able to modulate dopaminergic pathways by influencing directly or indirectly the activity of dopaminergic neurons through mechanisms either on the post- or pre-synaptic site of the neurons (Laviolette and Grace, 2006). However, the mechanisms by which dopaminergic and endocannabinoid signalling communicate with each other and the role of the dopamine D1 receptor positive neurons are still not clear (Terzian et al. 2011). But the colocalization of the CB1 receptor with the D1 receptor indicates that both types of receptors might functionally interact (Hermann et al., 2002).

Characterization of basic function and model of locomotion and motor functions

$CB1^{f/f;D1\text{-}Cre}$ mice were first tested for differences in basal functions such as body weight. Fig. 20 shows the results for a typical group of $CB1^{f/f;D1\text{-}Cre}$ animals as compared to their respective wild-type littermates. No differences in body weight were found between the genotypes, indicating that the loss of CB1 receptor on the dopamine D1 receptor expressing MSNs in the striatum has no major influence on the growth of the animals. Next, I tested $CB1^{f/f;D1\text{-}Cre}$ animals in different models of motor behaviour. In the open field, locomotion and motor functions were measured within a predetermined time period in both genotypes. $CB1^{f/f;D1\text{-}Cre}$ animals exhibited no basal difference in the locomotion as compared to wild-type mice in this model. A decrease in locomotor activity could be observed in a study of knock-out animals for the CB1 receptor that supports the role of the ECS on movement (Zimmer et al., 1999). But our results indicate that this decrease in locomotor activity does not depend on the modulation of MSNs. It is known that cannabinoid receptor agonists can increase movement at very low doses. This is followed by the appearance of rigidity and catalepsy with high doses (Sañudo-Peña et al., 2000). Thus, cannabinoids have a stimulating role for movement, which can be overridden at higher doses by the major modulatory actions of these compounds counteracting opposite systems such as the dopaminergic or GABAergic system. But no effect on locomotion was seen in $CB1^{f/f;D1\text{-}Cre}$ mice in a basal untreated state. General motor learning in the rotarod test was not changed either in these animals. No difference between the genotypes could be found in the time spent walking on the rod over all test sessions. However, the wild-type animals showed a significant increase of time spent on the rod between the first and the last day of experiment, showing that these mice were able to improve their performance in this test, while the same learning effect in the knock-out group was not significant and slightly decreased compared to their littermates. This indicates that there is a slight reduction in the motor learning ability of $CB1^{f/f;D1\text{-}Cre}$ animals. Psychomotor skill learning, as assessed in the rotarod test, involves cortical regions together with the striatum and cerebellum. An improvement in performance by learning is accompanied by changes in the striatum and motor cortex. ‘‘Fast’’ learning (improvement within the training session) involves a recruitment of task-related neurons in both brain structures, whereas the firing pattern is refined during the ‘‘slow’’ learning period (improvement between sessions) (Bilkei-Gorzo et al., 2005). As striatal projecting neurons are GABAergic and subthalamic projecting neurons are glutamatergic, the influence of both of these neurotransmitters together with the dopaminergic system is possible, but no effect could be detected in our animals indicating no direct influence of the loss of CB1 receptor on the dopamine D1 receptor expressing MSNs.

$CB1^{f/f;\ D1\text{-}Cre}$ animals show an unaltered behavioural response in models of anxiety

The models of anxiety used are based on the natural conflict of the rodents between the tendency to explore a novel environment and their aversion of a brightly lit and open area. Additionally, the elevated plus maze has another aversive characteristic, namely the height. The runways or "arms" of the EPM exhibit a specific height and openness (Carobrez and Bertoglio, 2005). The percentage of time which the animal spends in open arms and the percentage of entries to the open arms represent a measure of its anxiety. In this study, under basal conditions, no significant differences in the percentage of time spent in open arms and in the percentage of entries in open arms were detected between both genotypes. This fits with the results of the open field, where the time spent in the centre is an additional measure of anxiety. Neither in the open field nor in the EPM could I detect a difference in anxiety between $CB1^{f/f;\ D1\text{-}Cre}$ animals and their wild-type littermates.

The second model of anxiety used in this study, was the light-dark (LD) test. The results revealed no significant differences in the entries into or the time spent in the lit compartment in a naïve state between both genotypes. $CB1^{f/f;D1\text{-}Cre}$ mice showed a slight increase of entries into the lit compartment and the time spent there as compared to the wild-type littermates, but this effect was not significant. Additionally, these results confirmed the results of the EPM and of the open field test. As mentioned above, all these procedures mostly reflect the conflict between exploration and avoidance of a novel environment. Thus, the reduction of exploratory behaviour in open arms of the EPM or in the lit compartment of the LD test is commonly associated with high emotionality or anxiety. Recently published results showed that $CB1^{f/f;D1\text{-}Cre}$ mice tested in the same paradigms also failed to show alterations in spontaneous exploration and locomotor behaviour (Terzian et al., 2011). These findings are in line with previous data showing no general anxiety-like phenotype in mice with a complete CB1 receptor deletion and with a specific CB1 receptor deletion on glutamatergic neurons under standard behavioural conditions and without treatment (Marsicano et al., 2002; Jacob et al., 2009). As mentioned above, the deletion of the CB1 receptor in $CB1^{f/f;D1\text{-}Cre}$ mice was only obtained in the striatum and layer VI of the cortex. In the striatum, approximately 30% of the CB1 receptor is still expressed. These neurons are D2 expressing MSNs and GABAergic interneurons (Monory et al., 2007). Several studies showed that the ECS plays a role in the processing of anxiety in different brain regions. Additionally, the basolateral complex of the amygdala (BLAC) is involved in the processing of emotion (Gallagher and Chiba, 1996). Beside this region, CB1 receptor is also expressed at high levels in other parts of the brain, which play a role in anxiety processing, e.g. hippocampus, and prefrontal and anterior cingular cortex (Herkenham et al., 1990, 1991; Marsicano and Lutz, 1999; Katona et al., 2001; Hájos and Freund, 2002). In the amygdala, levels of anandamide and 2-AG are increased in

response to anxiogenic stimuli (Marsicano et al, 2002). In addition, inhibition of FAAH and anandamide reuptake leads to reduced anxiety-like behaviour in rats (Kathuria et al., 2003). A high density and/or functional activity of CB1 receptor was reported not only in the striatum, but in the limbic system and other brain areas related to emotional responses as well, such as hypothalamus and hippocampus (Herkenham et al. 1991). In the $CB1^{f/f;D1\text{-}Cre}$ mutants, CB1 receptor in all other brain regions except striatum is present and can interact with all other known neurotransmitter pathways involved in emotional behaviour. The colocalization of cannabinoid and other receptors, such as opioid receptors in several limbic structures including the nucleus accumbens and the amygdala (Mansour et al., 1995), raises the hypothesis of an interaction between opioid and cannabinoid systems to control anxiety-like responses (Berrendero and Maldonado, 2002). Due to the fact that a functional endocannabinoid signalling in all these regions is still present in our mutants, an unaltered anxiety-response can be expected. But it might be interesting to test the response of the $CB1^{f/f;D1\text{-}Cre}$ animals after treatment with different doses of Δ^9-THC and to see whether the animals show an altered behavioral outcome in response to high or low doses.

Models of recognition memory and learning revealed an increase of exploration following the deletion of GABAergic CB1 receptor and a decrease of exploratory behaviour following the deletion of glutamatergic CB1 receptor

Together with Martin Häring, a doctoral student in the Lutz` laboratory, I tested the behaviour of the $CB1^{f/f;D1\text{-}Cre}$ animals in models of recognition memory and learning. Additionally, I decided to test two of our other mouse lines, $CB1^{f/f;dlx5/6\text{-}Cre}$ and $CB1^{f/f;NEX\text{-}Cre}$, in order to show how the deletion of the CB1 receptor from forebrain GABAergic and cortical glutamatergic neurons, respectively, influences the behavioural outcome regarding animate and inanimate exploration. Starting with the novel object recognition task (NORT), I could show that the $CB1^{f/f;D1\text{-}Cre}$ mouse line did not show any significant differences regarding the exploration of the objects. All animals explored the A objects with the same duration during the training period (Fig. 28) and were able to distinguish between novel and familiar object in the retention sessions after 2 and 24 hours (Figs. 29, 30), indicating that the deletion of the CB1 receptor from dopamine receptor D1-expressing GABAergic striatal MSNs has no effect on exploration in this model, and did not alter learning. The same results were found in the sociability paradigm and the resident-intruder test. The $CB1^{f/f;D1\text{-}Cre}$ mouse line did not display any significant differences in regard to exploratory and aggression behaviour. In contrast, by using the other two conditional CB1 receptor mutant mouse lines, $CB1^{f/f;dlx5/6\text{-}Cre}$ and $CB1^{f/f;NEX\text{-}Cre}$, I was able to show that the deletion of the CB1 receptor from forebrain GABAergic and cortical glutamatergic neurons, respectively, resulted in an opposite behavioural outcome regarding animate and inanimate exploration (Häring et al., 2011). These findings suggest a regulatory

function of the ECS in cortical GABAergic and glutamatergic circuits to prevent neuronal and behavioural imbalance.

Mice lacking the CB1 receptor on glutamatergic neurons displayed a decreased exploratory behaviour, both in animate and inanimate interaction. A similar decrease in object and social exploration was found in earlier studies, which was related with increased fear (Lafenêtre et al., 2009; Jacob et al., 2009). In the present study, the decrease in exploration was seen when the mouse was exposed to a social interaction partner and/or to an object (Fig. 38), and seemed to be independent of novelty. This phenotype was also visible in the resident-intruder test (Fig. 41). However, the decreased social investigation was mainly based on a lower exploration during the first 5 min interval, a period important for information gathering (Fig. 41, left panel). The anxiogenic-like behaviour of these mutants can also explain the significantly higher aggression level found in the resident-intruder test (Fig. 41, right panel), a behaviour which was also observed in complete CB1 receptor knock-out animals (Martin et al., 2002). The age and strength of the intruder as compared with the resident is highly important (Thor and Flannelly, 1976). In this case, the intruders were weaker and should not be regarded as a threat. Therefore, it can be argued that the deletion of the CB1 receptor from glutamatergic neurons might result in an inadequate aggressive response, suggesting an important role of the CB1 receptor on this neuronal population in aggression. CB1 receptor in cortical GABAergic interneurons appears to mediate an opposite behaviour. While $CB1^{f/f;D1\text{-}Cre}$ animals (CB1 receptor loss primarily in the striatum), did not reveal any significant difference as compared to wild-type littermates (Fig. 34, 35), I observed that $CB1^{f/f;dlx5/6\text{-}Cre}$ mice (lacking CB1 receptor from striatal MSNs *and* from cortical GABAergic interneurons), showed an increase in animate and inanimate exploration (Fig. 36). Accordingly, increased investigatory behaviour toward novel food and object was previously observed in the $CB1^{f/f;dlx5/6\text{-}Cre}$ mice (Lafenêtre et al., 2009). Interestingly, $CB1^{f/f;NEX\text{-}Cre}$ control wild-type animals displayed an increased aggressive behaviour in the resident intruder paradigm as compared to the other wild-type controls of $CB1^{f/f;dlx5/6\text{-}Cre}$ and $CB1^{f/f;D1\text{-}Cre}$ mouse lines. This elevated aggression might be explained by the fact that both the wild-type and knock-out littermates were housed together during growth. In case of the $CB1^{f/f;NEX\text{-}Cre}$ line, the modulated social behaviour of the $CB1^{f/f;NEX\text{-}Cre}$ knock-out animals might have an effect on their wild-type littermates.

Taken together, these results suggest an anxiolytic-like function of the CB1 receptor on glutamatergic neurons and an anxiogenic-like function of the CB1 receptor on GABAergic interneurons. However, a generalized conclusion on the involvement CB1 receptor on cortical glutamatergic neurons in anxiety is not yet possible, as under the experimental conditions, the open field test was not congruent with this notion. Neither $CB1^{f/f;NEX\text{-}Cre}$ nor $CB1^{f/f;dlx5/6\text{-}Cre}$ mutants spent a different period of time in the more aversive centre zone as compared to

their respective wild-type littermates (Tab. 4), which could be caused by the fact that the used open field might be too small to induce anxiety. In addition, studies with these animals on the EPM did not reveal any changes either (Jacob et al., 2009; Ruehle et al., 2012). Also, levels of corticosterone under basal and stressful conditions were found to be similar between mutant and wild-type controls in both mutant lines (Steiner et al., 2008). Thus, it seems that an exploratory stimulus, such as an object or interaction partner, is required to induce a phenotype in these mice. An alternative explanation for the observed differences can be alterations in spontaneous locomotor activity. In fact, I observed that both the $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ animals displayed changes in the distance moved, namely a decrease and increase, respectively. It seems unlikely that the difference in locomotion was the driving force underlying the exploration phenotypes, as the mutants, in contrast to the variation in animate and social investigation, did not always display the locomotor alterations (Tab. 4). I assume that a certain context (e.g. handling threshold, exploratory stimulus) is required for a detectable locomotion phenotype in these mutant lines. The case seems to be similar for the general investigatory drive. Thus, the clear differences in exploring object or interaction partner are not mirrored by the findings in the open field test, where I was not able to detect any alteration in the time spent in the more aversive centre region (Tab. 4). This outcome is supported by other studies with these mutant lines, where a behavioural change is only detectable in the presence of a certain stimulus or pharmacological modification of the ECS (Lafenêtre et al., 2009, Jacob et al., 2009). A further explanation for the behavioural differences might be memory alterations in the respective knock-out animals. However, this might only account for the results of the $CB1^{f/f;NEX\text{-}Cre}$ mutants, as all other animals, independently of line and genotype, displayed a similar memory and recognition performance. Especially after 24 hours, all mice of the $CB1^{f/f;dlx5/6\text{-}Cre}$ and $CB1^{f/f;D1\text{-}Cre}$ mouse lines were able to recognize and distinguish strongly between familiar and novel objects (Tab. 4). The low discrimination index to the familiar object after a two hour interval in several groups, however, is unexpected and cannot be explained at this point. Only $CB1^{f/f;NEX\text{-}Cre}$ failed to show a clear preference towards the novel object in both retention sessions, indicating a memory deficit. This fact is difficult to interpret because of the low overall exploration for this mouse line, which is true for all three sessions of the NORT, as well as for the other behavioural paradigms. Of special interest is the altered behaviour of the knock-out mice in response to the novel objects. While wild-type littermates displayed a constant interest in the novel objects (A, B, C right), the $CB1^{f/f;NEX\text{-}Cre}$ animals showed a steadily decreasing exploration of the objects over the three sessions (Fig. 31, 32, 33, right panel). For both genotypes, such a decrease was seen regarding the exploration of the familiar objects (A left), which is not surprising, as the novelty of this object strongly decreased with each session. Thus, the $CB1^{f/f;NEX\text{-}Cre}$

mice appeared to respond to the familiar and novel object in a similar way, suggesting rather a habituation to the context than a memory deficit. Nevertheless, a final conclusion cannot yet be drawn. As mentioned above, all groups, independently of the line and the genotype, showed a stronger preference for the social interaction partner than for the object in the sociability test (Tab. 4). This behaviour was expected, as animals normally prefer social over non-social contacts (Moy et al., 2004). Surprisingly, I could not detect a significant preference towards the novel interaction partner in the social novelty phase (Tab. 4). While this preference was observed in several lines (Moy et al., 2004), in my experiments it was only detectable in the $CB1^{f/f;dlx5/6\text{-}Cre}$ mice (Fig. 36). This finding could indicate that social discrimination is impaired in these mutants. However, comparable results from other studies suggest that a strong social preference does not necessarily predict a strong preference for social novelty. As a matter of fact, two different components of social behaviour were postulated to underlie sociability and social novelty. In addition, life history and development are responsible for lower or higher novelty preference (Moy et al., 2009). The strong differences observed in the $CB1^{f/f;dlx5/6\text{-}Cre}$ and $CB1^{f/f;NEX\text{-}Cre}$ animals in respect to their wild-type littermates might be explained by anxiolytic and anxiogenic responses to novelty. Nevertheless, the ECS has also been shown to be involved in learning and memory function (Moreira and Lutz, 2008; Marsicano and Lafenêtre, 2009), which should be kept in mind. It is likely that both anxiety and memory components function together in these paradigms, but to solve this issue it would require further investigations using other behavioural paradigms, e.g. fear conditioning task, novelty-induced grooming test or the Barnes maze test.

The increase of exploration following the deletion of GABAergic CB1 receptor and the decrease of exploratory behaviour following the deletion of glutamatergic CB1 receptor may explain the contradictory findings using the CB1 receptor agonist Δ^9-THC, which, especially during adolescence, led to a decreased social interaction in rats or the use of the inhibitor of anandamide degradation URB597 and the putative anandamide reuptake inhibitor VDM11, which resulted in increased social play behaviour (Trezza and Vanderschuren, 2008a; 2008b; 2009). I suggest that increased or decreased exploratory drive as response to cannabinoid treatment depends on the predominant modulation of either GABAergic or glutamatergic CB1 receptor, i.e. the activation of GABAergic CB1 receptor decreases exploration, while the activation of glutamatergic CB1 receptor leads to an increased investigatory drive. Thus, the decreased exploration induced by chronic and systemic activation of the ECS with Δ^9-THC might be due to the exogenous activation of the CB1 receptor in GABAergic interneurons (O´Shea et al., 2004; Schneider et al., 2008; Trezza and Vanderschuren, 2008a). The increased exploratory profile after inhibition of anandamide degradation or reuptake could be explained by a specific on-demand activation of the CB1 receptor on glutamatergic

neurons (Trezza and Vanderschuren, 2008a). On the other hand, the increased animate and inanimate interaction as a result of the complete deletion of the CB1 receptor might be caused by the increased GABAergic drive (Jacob et al., 2009; Haller et al., 2004). It seems that GABAergic drive is the predominant factor for the behavioural outcome, when the ECS is activated or blocked in a chronic manner. This makes the increased social interaction after URB597 treatment even more interesting, as in this case, the glutamatergic drive seems to be the predominant component. To test this hypothesis, $CB1^{f/f;NEX\text{-}Cre}$ or $CB1^{f/f;dlx5/6\text{-}Cre}$ knock-out animals have to be injected with respective drugs, e.g. URB597 or VDM11 in comparable doses and tested in behavioural paradigms. Similar contradictory results were observed in pharmacological studies on anxiety and stress levels after cannabinoid administration, both studies being strongly involved in investigatory and exploratory drive (Hill and Gorzalka, 2005; Viveros et al., 2005). The opposite effects might also be based on changes in cortical GABAergic or glutamatergic transmission. Therefore, depending on its specific spatiotemporal activation within neuronal circuits, the ECS can act as a major ‘‘bi-directional’’ neuromodulator (Ruehle et al., 2012; Viveros et al., 2005).

The results are also interesting in respect to some human disorders, which are associated with inappropriate exploratory drive. Thus, a direct and indirect relation between these disorders and a dysregulation of GABAergic and/or glutamatergic transmission can be proposed. In animal models for autism, modulation of GABAergic transmission seems to be important (Chao et al., 2010; Sala et al., 2011). The induction of schizophrenia-like symptoms by administration of the NMDA receptor antagonist phencyclidine revealed an increase of glutamatergic and a decrease of GABAergic signalling in the prefrontal cortex (Amitai et al., 2011). Interestingly, the effects of phencyclidine could be blocked by CB1 receptor antagonist treatment (Del Arco et al., 2010). It was further shown that down-regulation of cortical glutamatergic drive resulted in an increase in dopamine levels and a hyperactive phenotype, which could be blocked by cortical GABA receptor activation (Guidali et al., 2011). These findings indicate a cortical control in these neuronal disorders, caused also by imbalanced GABAergic and glutamatergic transmission, a mechanism also suggested by our findings. Recent publications even suggest glutamatergic, instead of dopaminergic transmission to be the major factor of schizophrenia (Javitt, 2010).

In conclusion, the results in this part of the study indicate an opposite role of the ECS in cortical GABAergic and glutamatergic neurons in the regulation of exploration, but not based on the striatal MSNs alone. Hence, further investigations along this line should detail the diverse effects of cannabinergic drugs on investigatory behaviour. As investigatory drive is often associated with impulsive behaviour, studies using appropriate paradigms relevant to impulsivity would be of great interest. Lastly, in future studies, the regulatory properties of the ECS

on cortical excitatory and inhibitory drive should be exploited in regards to psychiatric disorders, opening up a therapeutic avenue to restore a possible cortical imbalance pharmacologically.

$CB1^{f/f;D1\text{-}Cre}$ mouse line shows a reduced susceptibility to the addictive effects of drugs in the models of addiction

Conditioned place preference (CPP) is one of the most popular experimental protocols used to measure drug reward in laboratory animals. Drug addiction is regarded as a chronic relapsing brain disorder characterised by neurobiological changes leading to compulsive drug seeking and drug taking despite serious negative consequences, and by the loss of control over drug use (Camí and Farré, 2003). Addiction integrates different behavioural and neurobiological processes. All drugs of abuse, such as nicotine, Δ^9-THC and cocaine, produce reinforcing effects accountable for the initiation of the addictive disorder (Maldonado et al., 2006). Before starting the experiments with the $CB1^{f/f;D1\text{-}Cre}$ mouse line, I tested the model itself to find the appropriate dose of nicotine in C57BL/6N mice. Merritt et al. showed in 2008 that the ECS plays a role in nicotine reward and dependence in $CB1^{-/-}$ animals and in age-matched C57BL/6J mice. The genetic deletion or pharmacological inhibition of CB1 receptor blocked the rewarding properties of nicotine CPP. Nicotine produced rewarding effects in the CPP paradigm in wild-type mice, but failed to produce any preference response in $CB1^{-/-}$ mice, regardless of the dose. Additionally, they could show that the CB1 antagonist rimonabant dose-dependently blocked nicotine CPP in C57BL/6 mice (Merritt et al., 2008). The conditional mutant $CB1^{f/f;D1\text{-}Cre}$ mouse line offers an interesting tool to gain more insight into the influence of the CB1 receptor and the ECS on addiction. Therefore, I compared the behaviour of the $CB1^{f/f;D1\text{-}Cre}$ mice and their wild-type littermates before and after the conditioning phase by using of a 0.5 mg/kg dose of nicotine, similar to Merritt and colleagues.

The dose of nicotine (0.5 mg/kg) significantly increased the time spent in the drug associated compartment of the wild-type animals, while this effect was absent in $CB1^{f/f;D1\text{-}Cre}$ mice (Fig. 45). These results indicated that the rewarding effects of nicotine are not as strong in the $CB1^{f/f;D1\text{-}Cre}$ animals as in wild-type animals. But the lack of a significant difference between the wild-type and the knock-out animals on the test day and the increase of the time spent in the drug-associated compartment (measure of conditioned preference) in both genotypes, urged me to repeat the experiment. Fig. 46 presents the results of the second experiment. There, I could find neither a significant increase in time spent in the drug-associated compartment for all trials nor any effect at all in the wild-type animals. Contrarily, the knock-out animals showed an increase in time spent in the drug-associated compartment, but this effect was not significant. This finding raised the question, whether or not the $CB1^{f/f;D1\text{-}Cre}$ mice

showed addiction to nicotine. It might be possible that the deletion of CB1 receptor in striatum does not influence this process. Therefore, I tested an additional batch of CB1 receptor null mutant mice ($CB1^{-/-}$) in order to find out whether this discrepancy in the results is caused by the conditional deletion of the CB1 receptor and whether I am able to repeat the results obtained by Merritt et al. (2008). But again, the results of this CPP were not in line with my expectations that the $CB1^{-/-}$ mice are not showing an effect to nicotine. Both genotypes showed a strong and significant increase in time spent in the drug-associated compartment, indicating no alteration by the CB1 receptor deletion in nicotine addiction. As this experiment was not in line with the experiment of Merritt et al. (2008), I searched for differences which might have caused the discrepancies. Analysis of the individual behaviour of each animal revealed a strong divergence in the behaviour of the mice (Fig. 48). Based on these results and the difference in background between this study and the Merritt study, I decided to evaluate strain differences as the next step. Strain differences in behavioural experiments can have a strong influence on the results. Crawley et al. (1997) reviewed that background genes from the parental strains may interact with the mutated gene, in a manner which could severely compromise the interpretation of the mutant phenotype and showed that C57BL/6 animals from a different background differed strongly in several behavioural paradigms, e.g. locomotor activity and sensitivity for drugs. More recently, it was shown how different the pain sensitivity behaviour of C57BL/6N and C57BL/6J can be or how strongly the laboratory environment can affect mouse strain differences in behavioural phenotypes (Bryant et al., 2008).

Two additional CPP experiments with both C57BL/N and C57BL/J background strains were performed and showed a significantly stronger and more homogenous response to nicotine for the C57BL/6J animals (Fig. 49, 50). This result was repeated with another model of addiction. One batch of each background strain was tested in the conditioned place aversion paradigm. C57BL/6N mice showed a weaker aversion phenotype and significantly reacted only to blockade of the 10 mg/kg/d dose of nicotine (Fig. 62), while C57BL/6J animals showed a significantly stronger reaction to the blockade of both nicotine doses (Fig. 63). Thus, I decided to cross both strains to obtain a F1 hybrid strain called C57BL/6NJ. After performing another CPP experiment, I could detect that C57BL/6NJ mice showed a reduced response to nicotine, similar to the original C57BL/6N phenotype (Fig. 51), but still had a better drug response than the C57BL/6N animals themselves, comparable with mice of the C57BL/6J background.

These obvious strain differences led to the decision to backcross our conditional mutant $CB1^{f/f;D1\text{-}Cre}$ mouse line and the null mutant $CB1^{-/-}$ mouse line for more than ten generations into the background of the C57BL/6J strain. With the help of the resulting conditional mutant

J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line and the null mutant J-CB1$^{-/-}$ mouse line I wished to gain more insights into the background differences regarding the addictive response to drug exposure.

Gene array and validation of candidate genes

During the time period of backcrossing I processed another CPP experiment with repeated nicotine injections. For this experiment, mice on C57BL/6N background were used to evaluate the effect of nicotine both on distinct behaviour and on gene regulation in the striatum. Fig. 51 shows that nicotine increased the time spent in the drug-associated compartment in CB1$^{f/f;D1\text{-}Cre}$ mice and in the wild-type animals as compared to the time spent in this compartment before the conditioning. But this effect was only significant for the wild-type mice. Based on their individual behaviour and drug injection, I separated both genotypes into saline-treated, nicotine-treated with aversion and nicotine-treated mice with preference. I prepared punches from their striata, processed the mRNA and sent these samples to the Institute for Translational Oncology (TRON) facility in order to perform a GeneChip Mouse Genome 430A 2.0 Array from Affymetrix.

Unfortunately, I could not find any significant false discovery rates (FDRs), a measure for the expected proportion of false positives among all significant hypotheses. This might be due to the fact that the deletion of CB1 receptor in the striatum affected a relatively small subset of all existing cells in the striatum: CB1 receptor expression is reduced but not absent in the striatum, with less than 30% of neurons still containing mRNA of the receptor in the caudate putamen and approximately 50% loss of CB1 receptor expression in the ventral striatum (Monory et al., 2007). With the gene array, I tested the expression changes in all available cells, thus, the effects based on alterations in the MSNs might be lost between the expression changes in all other cells. But as the data for the control gene *cnr1* was in the expected 30-50% range, I decided to go on with this study. Validation with qPCR showed that only four of the nine candidate genes display significant fold changes, either in untreated animals or in the control animals, treated with vehicle. Thus, I assumed that the five other candidates I tested were false positive results of the gene array experiment. More importantly, none of the candidate genes showed any significant expression difference between the genotypes in the groups of nicotine treated animals, neither in the group with aversion behaviour nor with preference behaviour, implicating that repeated nicotine injections had no effect on gene alterations in the striatum of CB1$^{f/f;D1\text{-}Cre}$ animals. This could be due to the fact that CB1 receptor positive cells represent only a small amount of all cells in the striatum. Unfortunately, I was not able to sort out and measure only the needed cell type. Additionally, it might be possible that four repeated nicotine injections of this low dose was not enough to cause gene expression alterations in the mice. As there were no effects seen in the nicotine treated

groups, I hypothesised that the significant fold changes for the candidate genes *pttg1*, *klf4*, *egr2* and *calml4* might be caused by the effect of the injection itself, indicating a general stress-induced effect on gene alteration.

The pituitary tumor transforming gene 1 (*pttg1*), coding for a transcription regulator in the nucleus of the cells, showed no differences between naïve wild-type and knock-out animals of $CB1^{f/f;D1\text{-}Cre}$ mouse line. But repeated vehicle injections during a CPP experiment caused a strong up-regulation in wild-type and a strong down-regulation of this gene in knock-out mice. As characterised by Wang and Melmed (2000), *pttg1* seems to have a role in tumorigenesis because of its expression in several tumor cell lines, but not in normal, healthy tissues. More recent publications showed a Pttg1-specific network in which *pttg1* is tightly linked to several genes, one of which is *rasd1* (a dexamethasone-induced small GTPase; Lum et al., 2006). Like *pttg1*, *rasd1* is regulated by estrogen and is associated with the circadian rhythm (Van Gelder 2004). Thus, the observed effect in the knock-out animals after vehicle injections can be caused by injection stress or maybe linked to differences caused by hormonal alterations, which could not yet be shown in this mouse line. The Kruppel-like factor 4 (*klf4*) is a transcription factor in the nucleus of the cells. There is no *klf4* deficient mouse model available for behavioural analysis, as $Klf4^{-/-}$ mice die shortly after birth due to defect in epithelial differentiation and barrier formation (Katz et al., 2002; Segre et al., 1999). It could be demonstrated *in vitro* and *in vivo* that in the CNS *klf4* expression increases in microglial cells with time upon lipopolysaccharide (LPS, an endotoxin) stimulation. Additionally, it was shown that *klf4* is involved in the up-regulation of proinflammatory cytokines and key inflammatory mediators including iNOS and Cox-2 and that *klf4* interacts with pNF-κB upon LPS treatment for its pro-inflammatory activity (Kaushik et al., 2010). Like *pttg1* and *klf4,* the third candidate gene early growth response 2 (*egr2*) is a transcription factor. It was shown that the ERK pathway is partially under the control of dopamine D1 receptor, which induces *fos*, *fosB*, *egr1* and *egr2* transcription (Benturquia et al., 2008). Additionally, the absence of *egr2* in forebrain neurons seems to facilitate certain forms of learning and memory and reinforces the notion that *egr1*, *egr2* and *egr3* might have different functions in the adult mouse brain and antagonistic functions (Poirier et al., 2008). De Steno and Schmauss (2008) showed an induction of *egr2* expression in areas of the medial prefrontal cortex of mice performing an attention set-shifting task, but not in a spatial working memory task. In this study, the authors detected no induction of *egr1* and *egr3* in the same brain structures in the attention-set-shifting task. These observations again suggest that *egr* family members might be differentially induced during specific cognitive tasks. In this study, *egr2* and *klf4* show a significant mRNA down-regulation in the striatum of naïve knock-out mice of the $CB1^{f/f;D1\text{-}Cre}$ mouse line versus their wild-type littermates. This effect is inversed in the saline-treated animals during a CPP experiment

indicating either a stress induced regulation of the genes or cognition alterations in the knock-out animals. For *klf4*, possible inflammatory processes caused by the repeated injections might also be assumed. The calmodulin-like 4 gene (*calml4*) is not well characterised yet. It seems to take part in Ca^{2+} binding and is able to interact selectively and non-covalently with Ca^{2+} (www.informatics.jax.org). Analysing by qPCR did not reveal significant differences in gene expression of *calml4*, but a tendency for the down-regulation as seen for *klf4* and *egr2*.

After completing the backcrossing of the $CB1^{f/f;D1\text{-}Cre}$ mouse line for more than ten generations into the C57BL/6J background, I repeated the CPP experiment. Fig. 52 revealed that both genotypes showed significant changes in time spent in the drug-associated compartment. Nicotine significantly increased the time spent in the drug-associated compartment in J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mice and in the wild-type animals as compared to their time spent in this compartment before the conditioning. There was no significant difference between the genotypes. All animals in C57BL/6J background responded equally to nicotine. Therefore I assume that the effect I saw in the first CPP in the $CB1^{f/f;D1\text{-}Cre}$ was not caused by the lack of CB1 receptor in D1-expressing MSNs, but an effect caused by the genetic background. The significantly lower response of $CB1^{f/f;D1\text{-}Cre}$ to nicotine was completely abolished in J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mice, confirming to the results found in both background strains (Fig. 49, 50).

Similar to the gene expression data obtained with the $CB1^{f/f;D1\text{-}Cre}$ mouse line, I also evaluated gene expression changes in the J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mouse line. I aimed at testing whether the observed effects of alterations in the candidate genes were due to the influence of stress or, similar to the behavioural results, caused by the influence of the used background strain. Analysis of the results for the control gene *cnr1* revealed a significant difference in expression between the wild-types of both conditional mutant lines. As the mutants of both lines showed the expected reduction of CB1 receptor in the striatum, I assume that this difference was due to the age of the used tissue. The brains of the $CB1^{f/f;D1\text{-}Cre}$ mouse line were stored at -80°C for more than 10 months. This might have caused mRNA degeneration in the tissue. For the other candidate genes, I could not repeat the results for the stress-induced changes found in the $CB1^{f/f;D1\text{-}Cre}$ mouse line. Untreated animals of both mouse lines showed the same gene expression for *pttg1*, but the significant reduction of *pttg1* in saline treated knock-outs could not be found in the J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mouse line. Additionally, I could not find the significant decrease in the fold change of *klf4* and *egr2*, revealed in the $CB1^{f/f;D1\text{-}Cre}$ animals but not in the J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mice. However, for both genes, I saw that the vehicle injections changed the expression in both lines in different ways. Wild-types of the J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ mouse line showed an increased fold change for *klf4* and *egr2* as compared to the wild-types of $CB1^{f/f;D1\text{-}Cre}$ mouse line, but no effect could be seen for *klf4* in the knock-outs of either lines, while for

egr2 a non–significant increase could be observed in the mutants of both mouse lines. As the tendencies in gene expression for *egr2* were similar in both mouse lines, I assume that *egr2* is a gene which seems to be linked to stress and not to the genetic mouse strain influences alone. The general larger effect on gene expression might be explained by the higher pain sensitivity in C57BL/6J mice (Bryant et al., 2008). Higher pain sensitivity is able to cause a larger stress reaction to the repeated injections in the model of addiction. Because of age-related tissue alterations and the different number of animals used in the experiments, an exact repetition of the results was not possible. I assume that this is also true for *klf4*. However, the genotype differences in gene expression found for *pttg1* in the striatum of CB1$^{f/f;D1\text{-}Cre}$ animals could not be seen in the striatum of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice, indicating that the first effect found was based on the genetic influence of the background strain C57BL/6N and got lost during backcrossing to the other genetic background.

The results gained in both mouse lines for *calml4* cannot be easily interpreted. The results shown in Fig. 89, present a completely different pattern for the gene expression in both mouse lines. While *calml4* is reduced in the CB1$^{f/f;D1\text{-}Cre}$ mouse line in naïve mutants and up-regulated in saline treated mutants, it showed the opposite effect in the wild-type mice. Naïve knock-out animals of the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line showed an already high gene expression of *calml4* as compared to their wild-type littermates. This gene expression is strongly down-regulated after vehicle treatment, while no differences can be observed in the wild-type littermates. As the effects of *calml4* in the brain are still not well known at the moment, I propose a general different expression of this gene depending on the genetic background of the conditional mutant lines.

Models of addiction and the effect of the genetic background

Additionally, I evaluated the behavioural response of the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line to cocaine in the model of CPP and compared this to the behaviour of our CB1 receptor null mutant J-CB1$^{-/-}$ mouse line. The CB1 receptor is predominantly found at pre-synaptic terminals of neurons (Marsicano and Lutz, 2006) and its activation leads to the decrease of the release of several neurotransmitters such GABA, glutamate, serotonin or dopamine (Szabo and Schlicker, 2005; Freund et al., 2003). Cocaine blocks the DAT, which is located on the pre-synaptic membrane of dopaminergic neurons, and clears dopamine from the synaptic cleft (Hyman and Malenka, 2001). It is known that in CB1$^{-/-}$ mice the loss of CB1 receptor reduces cocaine self-administration without differences in the extracellular levels of dopamine (Soria et al., 2005).

Therefore, I aimed at testing whether the conditional mutant J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line reacts differently to repeated cocaine administration as compared to the CB1 receptor null mutant J-

CB1$^{-/-}$ mouse line. As the D1 receptor is expressed in striatal GABAergic neurons and the Cre recombinase is expressed under the control of the regulatory sequences of dopamine D1 receptor, I hypothesised that Cre-mediated recombination can only take place in the projecting glutamatergic neurons. This fact might reveal a higher influence of glutamatergic neuronal subpopulations on addiction.

Both mouse lines (J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ and J-CB1$^{-/-}$ mouse line) were tested with 10 mg/kg cocaine. Additionally, only the J-CB1$^{-/-}$ mouse line performed a CPP with 20 mg/kg dose of cocaine. Both genotypes of both mouse lines showed a significant preference for cocaine (10 mg/kg) after CPP experiments, indicating no direct effect of the CB1 receptor on the addictive properties of cocaine. The results even revealed that J-CB1$^{-/-}$ animals reacted significantly stronger to cocaine as compared to their wild-type littermates. Additionally, a small tendency towards a significant difference could be detected in knock-out animals of the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line when compared with knock-outs of the J-CB1$^{-/-}$ mouse line. J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice seem to show a smaller preference for cocaine than J-CB1$^{-/-}$ mice and wild-type animals of both lines, indicating that the loss of the CB1 receptor only on MSNs of the striatum reduces the rewarding effect of cocaine administration. In contrast, the lack of CB1 receptor in all cells seems to enhance the addictive effect of cocaine as compared to wild-type animals and J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mice. This result is interesting as the saline-treated knock-out control animals of J-CB1$^{-/-}$ mouse line show no habituation to the conditioning chambers. A habituation to the conditioning chamber usually causes a general reduction of the time spent in the drug-associated compartment, because the drug-associated compartment is chosen for the natural aversion to this chamber in the pre test of the experiment. A saline-treated animal shows no drug effect and reduces the time in the naturally non-preferred chamber. If an animal is not able to habituate, it shows the same interest to this chamber as already seen in the pre test.

Testing the null-mutant mice with a higher dose of cocaine (20 mg/kg) did not reveal significantly different results as compared to the experiment with the lower drug dose. Also here, the mutant and the wild-type animals showed significant preference to the drug. I compared all mouse lines additionally for the hyperlocomotion effect caused by cocaine. Starting with the lower dose of cocaine in the J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ and J-CB1$^{-/-}$ mouse line, I detected an significant increase in the distance moved in all cocaine-injected animals compared to their respective vehicle treated littermates. Additionally, I saw another significant increase in the distance moved comparing the cocaine treated J-CB1$^{-/-}$ knock-out animals with their cocaine treated wild-type littermates and with the knock-out mice of J-CB1$^{f/f;J\text{-}D1\text{-}Cre}$ mouse line, pointing out that the complete loss of CB1 receptor potentiates the rewarding properties of cocaine. By using a higher dose of cocaine, I could still show the similar hyperlocomotional drug effect in

the J-CB1$^{-/-}$ mouse line, but this effect was even significantly increased with the higher dose. Until today, no publication has shown this kind of effect in CB1 receptor null-mutant mice. The few studies available have not supported a role of CB1 receptors in the primary reinforcing effects of cocaine in the J-CB1$^{-/-}$ mouse line. However, the CB1 receptor appears to be required for the incentive motivational effects of cocaine, as measured by self-administration under progressive-ratio schedules (Soria et al., 2005) and by reinstatement of extinguished cocaine self-administration (De Vries et al., 2001). The ECS seems to modulate cocaine addiction through neuronal circuits different from its acute effects on DA activity in the nucleus accumbens, which is a prominent substrate for drug rewarding effects (Soria et al., 2005).

In my experiments, the J-CB1$^{-/-}$ mouse line showed a distinct effect to cocaine. This effect is partially in agreement with the results of Miller et al. (2008). They showed that a high dose of cocaine (17 mg/kg) is able to induce cocaine place preference in naïve CB1 receptor deficient mice. In contrast to my results, Miller et al. could not observe place preference with a dose of 10 mg/kg cocaine in their mice. Only when the mice underwent a chronic unpredictable stress paradigm before the CPP protocol, these null-mutant mice developed preference to cocaine. This raises the question whether our animals were imposed by a chronic stress state. It might be possible that the handling prior to and during the experiment caused already a high stress situation, which led to their enhanced preference to cocaine in the CPP experiment. As already mentioned, different C57BL/6N substrains and the C57BL/6J strain differ in pain sensitivity or the laboratory environment can affect behavioural phenotypes strongly (Bryant et al., 2008). As pain sensitivity is higher in the C57BL/6J strain than in C57BL/6N, this effect might be present in the mutant lines from that genetic background as well. Additionally, exposure to stressful events triggers synaptic adaptations in many brain areas. Stress might also alter cannabinoid-receptor-mediated transmission in the brain. It was shown that social defeat stress, induced in mice by exposure to aggression, altered CB1 receptor-mediated control of synaptic transmission in the striatum (Rossi et al., 2008). Therefore, the loss of CB1 receptor might enhance the stress level of the animals used here.

A recent publication was able to show that the local administration of JWH133, a selective CB2 receptor agonist, dose-dependently inhibited intravenous cocaine self-administration, cocaine-enhanced locomotion, and cocaine-enhanced accumbens extracellular dopamine in wild-type and CB1 receptor knock-out mice, which suggests that brain CB2 receptor modulates cocaine reward and locomotor-stimulating effects, likely by a dopamine-dependent mechanism (Xi et al., 2011). I assume that a low density of CB2 receptor might be expressed on mesolimbic DA neurons. As the CB2 receptor is $G_{i/o}$ coupled, an activation of the CB2 receptor on DA neurons in the midbrain ventral tegmental area (VTA) may directly inhibit VTA dopaminergic neurons and decrease dopamine release in the nucleus accumbens, and

therefore inhibit intravenous cocaine self-administration and cocaine-enhanced locomotion, as observed by Xi et al. (2011). Although direct evidence of CB2 receptor expression in the mesolimbic DA neurons is currently lacking, functional CB2 receptor is found on other neurons. CB2 receptor mRNA is expressed on striatal GABAergic neurons in non-human primates (Lanciego et al., 2010) and activation of CB2 receptor inhibits GABAergic neurotransmission in the medial enterorhinal cortex of the rat (Morgan et al., 2009). Thus, CB2 receptor activation might cause the effect seen in the used animals.

The endocannabinoid system and the dopaminergic system are both involved in addiction (Valjent et al., 2002). Until today, it has not been clarified yet which neuronal mechanisms and circuits are exactly involved in these processes and how they are precisely affected by the ECS. However, nicotine produces its rewarding effects by stimulating mesolimbic dopaminergic transmission (Dani and De Biasi, 2001; Di Chiara, 2000), a common feature of all typical addictive drugs, while cocaine blocks the DAT on the presynaptic membrane of dopaminergic neurons, and thereby increases a blockage of synaptic dopamine (Hyman and Malenka, 2001). The activation of dopamine release by nicotine depends on a functional balance between excitatory and inhibitory inputs to the ventral tegmental area (VTA) dopaminergic neurons, in addition to the direct nicotine effects on dopaminergic neurons themselves (Mansvelder and McGehee, 2002). These dopaminergic neurons are not involved in the Cre-recombinase-mediated loss of CB1 receptor in $CB1^{f/f;D1\text{-}Cre}$ mice. Cocaine-induced phosphorylation and activation of extracellular signal-regulated kinase (ERK), through combined stimulation of dopamine D1 receptors and glutamate NMDA receptors, is restricted to D1-expressing MSNs in the dorsal striatum and nucleus accumbens core and shell (Bertran-Gonzales et al., 2008), where the CB1 receptor is lacking.

As striatal projections arise from GABAergic neurons and subthalamic projections arise from glutamatergic neurons, and both neuronal populations express the CB1 receptor, endocannabinoids are able to regulate inhibitory and excitatory inputs with the help of those neurotransmitter systems (Alexander and Crutcher, 1990). The ECS may also contribute to the regulation of this balance (Castane et al., 2005). Indeed, *in vivo* brain microdialysis studies have revealed that the CB1 receptor antagonist rimonabant (SR 141716) blocked nicotine-induced dopamine release in the shell of the nucleus accumbens (Cohen et al., 2002). Different nicotinic acetylcholine receptor (nAChR) subtypes modulate GABAergic and glutamatergic inputs to VTA dopaminergic neurons. While heteromeric nAChRs modulate GABA release, homomeric nAChRs influence glutamate transmission (Mansvelder and McGehee, 2002). Endocannabinoids are able to inhibit the function of homomeric nAChRs expressed in *Xenopus laevis* oocytes (Oz et al., 2004). Therefore, the activity of homomeric nAChRs could also be modulated by endocannabinoids *in vivo*, thus contributing to the regulation of the

rewarding properties of nicotine. Drug addiction is a complex behavioural and neurochemical process in which many neuroanatomical pathways and neurotransmitters are involved. This study supports the specific role of the ECS in the modulation of responses to nicotine and cocaine related to their addictive properties.

6.2. Behavioural characterization of NG2-EYFP mouse line

The NG2-EYFP mouse line was generated by Karram et al. (2008). This mouse line was primarily developed in order to facilitate studies on the function of NG2+ cells and to characterize these cells *in situ*. For this purpose, the enhanced yellow fluorescent protein (EYFP) ‘‘knock-in mouse’’ was generated (Karram et al., 2008). Heterozygous mice of this mouse line express the NG2 protein in all regions and at all ages studied by Karram and colleagues (2008). By inbreeding these mice, the NG2 null mutant mice were gained. At the cellular level, wild-type (+/+) and heterozygous (+/-) mice express the NG2 protein, while the homozygous mouse (-/-) lacks the NG2 protein. But *vice-versa,* NG2-EYFP heterozygous and homozygous mice express EYFP, while the wild-type animals lack any expression of EYFP. This study now aimed at testing wild-type (+/+) and homozygous (-/-) NG2-EYFP mice to explore the function of the NG2 protein in the CNS. By testing this mouse line in different behavioural models, I focused on characterization of the NG2 function *in vivo* for the first time.

Starting with the tests for the basic functions of these mice, I found no differences in the body weight, but already a significant decrease of reaction in the acoustic startle response test. Knock-out animals of the NG2-EYFP mouse line responded significantly less to an auditory stimulus of 95 dB. This result indicates a hearing disability in the knock-out animals. I did not test old animals and therefore can exclude an age-dependent hearing disability. In 2000, Bergles et al. demonstrated the existence of functional neuron-glia synapses in the brain (Bergles et al., 2000). Thus, the damage of these neuron-glia synapses due to the loss of NG2 protein might have altered Ca^{2+} permeability and synaptic transmission and changed the reactions of the animals to the auditory stimulus.

In the models of motor function and locomotion, I was not able to detect any significant differences between knock-out and wild-type animals. No phenotype could be found in the open field and rotarod test. The significant difference in the grip strength measurement was due to the age dependent decline of strength between young and old animals of both genotypes. This indicates that a loss of NG2 in the cells of the CNS does not interfere with the development of motor functions and locomotion changes.

Next, I tested the NG2-EYFP mouse line in the models of emotion. Similar to the behaviour seen in the models of motor function and locomotion, the mice of both genotypes showed no alterations in the EPM. But in the LD paradigm, I could detect a significant increase of the time spent in lit compartment for the NG2 knock-outs as compared to their wild-type littermates. As a trend, this effect was also found for the entries into lit compartment as well. Additionally, I could find a decrease in the floating time in the FST for the mutant animals. This indicates that a loss of NG2 causes an anxiolytic phenotype.

It was shown that a distinct subset of NG2+ glial cells in the hippocampus receive direct synaptic input from glutamatergic and GABAergic neurons (Bergles et al., 2000; Jabs et al., 2005). Additionally, Jabs et al. (2005) could show that NG2-positive glutamate receptor-expressing cells develop an unusual synaptic association with neurons. Therefore, it might be possible that NG2 glia act not only as a plastic progenitor pool for more differentiated cells, but might be able to form a unique glial network. The multiple PDZ domains of NG2 protein provide a wealth of potential sites for clustering NG2 with other structural and/or signalling molecules (Karram et al., 2005). In addition, the group of Prof. Trotter was able to show that NG2, GRIP and the AMPA receptor subunit GluRB, which are all co-expressed in glial progenitor cells, form a complex (Stegmuller et al. 2003). Therefore, the loss of NG2 in cells seems to have an influence on functional glutamate transmission. This effect raises the hypothesis that the clustering between NG2, GRIP and GluRB is altered and the uptake of glutamate decreased. Similar to the anxiolytic effect of CB1 on glutamatergic neurons (Häring et al., 2011), the loss of NG2 might change the glutamatergic structure and pattern not only in the glial network, but also in the neuronal network, causing an anxiolytic phenotype in the knock-out animals of the NG2-EYFP mouse line.

The results in both models of recognition memory and learning showed no differences between the genotypes of NG2-EYFP mice. Neither in the NORT nor in the MWM could I find differences in the performance between wild-type and knock-out littermates. Both groups were able to learn the task to find the hidden platform and to discriminate between new and familiar objects. The lack of NG2 protein seems to have no influence on the learning ability of the animals.

In the final experiment, I tested the NG2-EYFP mouse line in the model of chemically induced excitotoxic seizures, where the spreading of uncontrolled glutamatergic neurotransmission, induced by injection of the neuroexcitatory substance kainic acid, causes seizures in the animals. I observed a generally lower response to the drug in knock-out animals of the NG2-EYFP mouse line than in wild-type animals. This response strength of the NG2-EYFP mice was significantly reduced over the whole scoring time, indicating a protection of the mice through the loss of NG2 protein. As already discussed for the models of anxiety and the

difference in acoustic response, it seems that the loss of NG2 protein could change the complex between NG2, GRIP and GluRB. Therefore, it may alter the formation of neuron-glia synapses and change the effect of glutamate on GluRB and limit the danger of excessive excitatory activity in the animals. Bergles et al. reviewed that the expression of receptors with significant Ca^{2+} permeability such as AMPA receptors may increase the susceptibility of NG2 cells to excitotoxic injury (Bergles et al., 2010). This implicates on the other hand that a loss of the NG2 protein alters Ca^{2+} permeability and therefore decreases the susceptibility to excitotoxic damage.

7. Conclusion

7.1 CB1 receptor conditional mutant mice and the endocannabinoid system

Conditional mutant mouse lines are powerful tools to explore the role of the ECS to get mechanistic insights into behaviour. It is known that genes and environment are both essential and interdependent determinants of behavioural responses, thus, the generation and analysis of mutants and the comparison with wild-type controls is an excellent tool to study this interaction (De Sousa et al., 2006).

The aim of this study was to explore the functions of the CB1 receptor in the D1 dopamine receptor expressing neurons of the caudate putamen (striatum) by the use of a CB1 receptor conditional mutant line, $CB1^{f/f;D1\text{-}Cre}$, and to investigate how the CB1 receptor is involved in functions of locomotion, addiction and emotion. The experiments revealed interesting aspects how the decrease of CB1 receptor expression in the striatum of the knock-out animals affects the behaviour as compared to their wild-type littermates in different behavioural paradigms. Additionally, I evaluated the effect of the genetic background on the behavioural outcome in the CB1 receptor conditional mutant lines ($CB1^{f/f;D1\text{-}Cre}$ versus $J\text{-}CB1^{f/f;J\text{-}D1\text{-}Cre}$) as well as on CB1 null-mutant mouse lines ($CB1^{-/-}$ versus $J\text{-}CB1^{-/-}$) in relation to drug-induced reward. Not only the effect of genes on the behaviour of the animals, but additionally the effect of a particular behavioural paradigm combined with drug treatment on distinct gene expression was evaluated in the $CB1^{f/f;D1\text{-}Cre}$ mice with different genetic background. To this end, I tested the hypothesis whether the effect of different background strains is able to interfere with the addictive effects of nicotine and cocaine. Furthermore, in order to explore the effect of different neuronal subpopulations in the brain on recognition memory, learning processes and social behaviour, I tested the $CB1^{f/f;D1\text{-}Cre}$ and their wild-type littermates in comparison to $CB1^{f/f;NEX\text{-}Cre}$ and $CB1^{f/f;dlx5/6\text{-}Cre}$ and their respective littermates.

In this study, different behavioural models were tested. Here, it was shown that the CB1 receptor deletion in the striatum caused no differences in locomotor activity and motor learning as compared with wild-type controls. In the experiments regarding emotion-related behaviours, no significant phenotypic changes were observed either. Thus, the loss of CB1 receptor expression in the striatum does not affect locomotion and anxiety.

As the glutamatergic and GABAergic signalling pathways take prominent roles in anxiety, and as the CB1 receptor is expressed in these neuronal populations and modulate the release of glutamate and GABA, respectively, CB1 receptor function in these two neuronal populations of the forebrain was also investigated. I could show a major, but opposite role of the ECS in cortical GABAergic and glutamatergic neurons in the regulation of exploration and

social investigation, whereby loss of the CB1 receptor in glutamatergic neurons reduces exploratory behaviour and social investigation, and loss of CB1 receptor in GABAergic neurons increases these behaviours.

The ECS and the dopaminergic system are both involved in addiction (Valjent et al., 2002). Yet, it remains unclear, which neuronal mechanisms and circuits are exactly involved in this processes and how they are precisely affected by the ECS. The results showed that in both conditional mutant $CB1^{f/f;D1\text{-}Cre}$ and J-$CB1^{f/f;J\text{-}D1\text{-}Cre}$ as well as both CB1 null-mutant mouse lines $CB1^{-/-}$ and J-$CB1^{-/-}$, nicotine and cocaine treatment induced rewarding effects with all administered doses. But this behaviour was differently affected by the genetic background, the impact of stress onto the animals themselves and by gene expression.

The stimulation of dopamine release by nicotine depends on a functional balance between excitatory and inhibitory inputs to the VTA dopaminergic neurons, in addition to the direct nicotine effects on dopaminergic neurons themselves (Mansvelder and McGehee, 2002). Cocaine induces fast dopamine increases in striatal brain regions, which are responsible for the rewarding effects (Di Chiara and Imperato, 1988). Signal transduction via the activation of D1 receptor-expressing neurons and the inhibition of D2 receptor-expressing neurons is important in pharmacological effects of cocaine. The rate dependency of cocaine effect seems to relate to its fast activation of D1 receptor expressing striatal neurons in contrast to the much longer lasting deactivation of neurons expressing the D2 receptor (Luo et al., 2011). Additionally, Luo et al. could uncover a crosstalk between D1- and D2-expressing neurons in striatum when challenged with cocaine. Since striatal projections are GABAergic and subthalamic projections are glutamatergic, the ECS is able to regulate inhibitory and excitatory inputs with the help of these neurotransmitter systems. Furthermore, the ECS is involved in the modulation of several neuronal circuits and influences different neurotransmitter releases. Therefore, it can regulate distinct brain functions, even though the mechanism of regulation of the different neurotransmitter systems is not precisely understood. By modulating neurotransmission, the ECS takes part in the modulation of behaviour as well. The use of conditional mouse lines in different behavioural analyses can show which distinct brain region is involved in certain behaviour development.

7.2. Behavioural characterization of NG2-EYFP mouse line

The NG2-EYFP mouse line is not only a successful and useful tool to investigate the function of NG2+ cells and to characterize these cells *in situ*, but also to explore the role of these cells *in vivo* by their effects on the behaviour of the mice as well.

It is not precisely known how the NG2 protein is able to interact with presynaptic neurons in the CNS. This could possibly occur by a receptor that interacts with the LNS domains of the NG2 protein, but such a neuronal receptor has still not been found. By now, it is known that in the NG2-EYFP mouse line many EYFP-expressing cells are associated with neurons (Karram et al., 2008). Since AMPA and $GABA_A$ receptors are expressed on the cell body and processes of NG2 cells, the NG2-EYFP+ cell may adapt to the cell body of the neuron waiting for neurotransmitter release (Karram et al., 2005). Stegmuller et al. showed in 2003 that the NG2 protein is able to interact with AMPA receptors via a complex with the PDZ-protein GRIP. Furthermore, it was found that NG2, GRIP and the AMPA receptor subunit GluRB, which are all co-expressed in glial progenitor cells, form a complex (Stegmuller et al. 2003). Together with the behavioural results for the NG2-EYFP mouse line in the light-dark and forced swim test, it seems possible that the loss of NG2 protein in cells has an influence on functional glutamate transmission, because of the anxiolytic-like phenotype I observed in the knock-out animals. This phenotype raises the hypothesis that the clustering between NG2, GRIP and GluRB is altered, and the uptake of glutamate is decreased.

Additionally, I was able to show a significant decrease in the hearing ability of the knock-out animals. I associate this phenotype with the same possibility as mentioned above. Bergles et al. reviewed that the expression of receptors with significant Ca^{2+} permeability, such as AMPA receptors, may increase the susceptibility of NG2 cells to excitotoxic injury (Bergles et al., 2010). This in turn implicates that a loss of the NG2 protein, which seems to be important for the functionality of the neuron-glia synapses, alters Ca^{2+} permeability and therefore decreases the susceptibility to excitotoxic injury. But future experiments will have to substantiate this hypothesis.

8. Summary / Zusammenfassung

8.1 Summary

In the first part of this thesis, my work aimed at dissecting and exploring the functional involvement of the CB1 receptor, a major component of the neuronal endocannabinoid system (ECS), in various aspects of behaviour by using conditional mouse mutants lacking the CB1 receptor on different neuronal subpopulations. Different behavioural models were tested. I focused on the $CB1^{f/f;D1\text{-}Cre}$ mouse line, which lacks the CB1 receptor in dopamine D1 receptor-expressing neurons of the striatum. I showed that the loss of the CB1 receptor on these neurons did not affect basal neurological functions, weight, movement, exploration, social behaviour, anxiety and stress coping of the animals, but seemed to have a role in the development of addictive behaviour. Regarding the addictive behaviour towards cocaine, these conditional mutant mice showed a reduced susceptibility as compared to animals with CB1 receptor loss in all cells of the body and to genetically unchanged mice of both mouse lines.
Furthermore, this study also revealed an opposing involvement of the ECS in the regulation of exploration in relation to the loss of CB1 receptor in forebrain GABAergic and cortical glutamatergic neurons, but not in striatal neurons alone. Additionally, I was able to show the importance of the genetic background of mouse lines not only in the formation of a specific behavioural phenotype, but also on gene expression.
In the second part of this thesis, I focused on glial function and its influence on behaviour. A genetic deletion of the glial NG2 glycoprotein caused a hearing disability and reduced depression-like behaviour in the knock-out mice as compared with wild-type controls. Interestingly, they also showed reduced susceptibility to chemically induced excitotoxic seizures, suggesting a role of NG2 in the control of glutamatergic homeostasis, probably caused by structural changes in the neuron-glia synapses.

8.2. Zusammenfassung

Im ersten Teil dieser Doktorarbeit beabsichtigte meine Arbeit, die funktionelle Beteiligung des CB1 Rezeptors, einer Hauptkomponente des neuronalen Endocannabinoid-Systems (ECS), an der Ausbildung von verschiedenen Verhaltensphänotypen mit Hilfe von konditionalen Mausmutanten, denen der CB1 Rezeptor auf verschiedenen neuronalen Unterpopulationen fehlt, aufzuschlüsseln und zu untersuchen. Verschiedene Verhaltensmodelle wurden hierzu getestet. Dabei lag der Fokus dieser Arbeit auf der $CB1^{f/f;D1\text{-}Cre}$ Mauslinie, welche der CB1 Rezeptor auf den D1 Rezeptor exprimierenden Neuronen des Striatums fehlt. Ich konnte zeigen, dass der Verlust des CB1 Rezeptors auf diesen Neuronen keinen Einfluss auf basale neurologische Funktionen, Gewicht, Bewegung, Exploration, Sozialverhalten, Angst und Stressbewältigung der Tiere hat, jedoch eine Beteiligung an der Entwicklung von Suchtverhalten gegeben ist. Bei Betrachtung des Kokain-induzierten Suchtverhaltens zeigten die konditionalen Mausmutanten eine reduzierte Suchtanfälligkeit sowohl im Vergleich zu Tieren mit einem totalen CB1 Rezeptor Verlust in allen Körperzellen, als auch zu genetisch unveränderten Kontrollmäusen beider Linien.

Des Weiteren zeigen die Ergebnisse dieser Studie eine große, aber gegensätzliche Beteiligung des ECS bei der Regulation von Exploration in Abhängigkeit des Verlustes des CB1 Rezeptors auf GABAergen Neuronen des Vorderhirns und kortikalen glutamatergen Neuronen, jedoch nicht auf striatalen Neuronen alleine. Zusätzlich war ich in der Lage, die Wichtigkeit des genetischen Hintergrunds von Mauslinien nicht nur auf die Ausbildung von spezifischen Verhaltensphänotypen, sondern auch auf die Genexpression zu zeigen.

In dem zweiten Teil dieser Arbeit, in dem ich mich auf die Funktion von Gliazellen konzentrierte, wurden ebenfalls Mausmutanten in verschiedenen Verhaltensmodellen getestet. Ein genetisches Auslöschen des NG2 Glykoproteins in Gliazellen sorgt in den Knock-out Mäusen für ein schlechteres Hörvermögen und ein reduziertes Depressionsverhalten im Vergleich zu ihren Wildtyp-Kontrollmäusen. Interessanterweise zeigten diese Tiere auch eine reduzierte Empfänglichkeit bei chemisch induzierten epileptischen Krämpfen, was eine Rolle des NG2 Glykoproteins bei der Kontrolle der glutamatergen Homöostase vorschlägt, die wahrscheinlich durch Strukturänderungen der Neuron-Glia-Synapse verursacht wird.

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Abbreviations

aa	amino acid(s)
AC	adenylyl cyclase
ADHD	attention deficit hyperactivity disorder (disease)
AEA	arachidonoyl ethanolamide; anandamide
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid
ANOVA	analyses of variance
ASR	acoustic startle response (test)
bp	base pair(s)
°C	degree centigrade
CA	cornu ammonis
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CBD	cannabidiol
CBN	cannabinol
cDNA	complementary desoxy-ribonuclein acid
cm	centimetre
CNS	central nervous system
CPA	conditioning place aversion (test)
CPP	conditioning place preference (test)
DA	dopamine
Cre	cyclization recombination recombinase
Cx	connexin
DAS	dopaminergic system
DAG	diacylglycerol
DAGL	1,2-diacylglycerol lipase
DAT	dopamine reuptake transporter
DEPC	diethylpyrocarbonat
DG	diacylglycerol
DI	discrimination index
DNA	desoxy-ribonuclein acid
DTT	dithiothreitol
ECS	endocannabinoid system
EYFP	enhanced yellow fluorescent protein
EMT	endocannabinoid membrane transporter
EPM	elevated plus maze (test)
FAA	fully antagonised anaesthesia
FAAH	fatty acid amide hydrolase
FC	fold change
FDR	false discovery rates
Fig.	figure
FLAT	FAAH-like anandamide transporter
FST	forced swim (test)
g	gram
GABA	γ-aminobutyric acid
GAG	glycosaminoglycan
GFAP	glial fibrillary acidic protein
GPe	globus pallidus pars externa
GPi	globus pallidus pars interna
GRIP	glutamate receptor interaction partner

GSM	grip strength meter
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HIP	hippocampus
IL	interleucine
IP3	inositol-triphosphate
l	litre
kDa	kilo Dalton
KO	knock-out
LD	light-dark test
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
LNS	laminin G/neurexin/sex-hormone binding-globulin
loxP	locus of crossover in P1
lux	lux
M	molar
µg	microgram
µl	microliter
µm	micrometre
MAG	myelin associated glycoprotein
MAGL	monoacylglycerol lipase
$MgCl_2$	magnesium chloride
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
MM	master mix
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
MSNs	medium spiny neurons
MWM	morris water maze (test)
nAChR	nicotinic acetylcholine receptor
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NAPE	N-arachidonoyl phosphatidylethanolamide
ng	nanogram
NG2	nerve-glia antigen 2
NH_4OAc	amoniumacetat
nm	nanometre
nM	nanomolar
NMDA	N-methyl-D-aspartic acid
NORT	novel object recognition (test)
NTC	non template control
OD	optical density
OF	open field (test)
OPC	oligodendrocyte precursor cell
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	postsynaptic density protein-95, discs-large, zonula occludens-1
PEG	polyethylenglycol
PFA	paraformaldehyde
PFC	prefrontal cortex
PH	phosphoinositide hydrolysis

PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PLP	proteolipid protein
PNS	peripheral nervous system
PPN	pendunculopontine nucleus
qPCR	quantitative real-time polymerase chain reaction
rpm	revolutions per minute
RR	rotarod (test)
SDS	sodium dodecylsulfate
SNr	substantia nigra pars reticulata
STN	subthalamic nucleus
STR	striatum
SVZ	subventricular zone
Tab.	table
TBS	tris buffered saline
THC	Δ^9-tetrahydrocannabinol
TNT	tris sodiumchloride Tween20
TPH	tryptophan hydroxylase
TRPV1	vanilloid receptor type 1
URB597	anandamide degradation inhibitor
UTP	uridin-tri-phosphate
VDM11	putative anandamide reuptake inhibitor
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
VZ	ventricular zone
WIN 55212-2	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de)-1,4 benzoxazin-6-yl]-1-napthalenylmethanone
WT	wild-type
2-AG	2-arachidonoylglycerol

Gene	Foldchange		Groups						
	wt u - ko u	wt u - wt c	ko u - ko c	wt c- ko c	wt p - ko p	wt a - ko a	wt c - wt p	wt c -wt a	wt p - wt a
	basal phenotype	stress induced	nicotine induced changes on phenotype				nicotine induced changes on genotype		
Pttg1	9 fold	4.5 fold	274 fold	10757 fold	22 fold	9505 fold	915 fold	2 fold	510 fold
Nptx1	2 fold	1.4 fold	1 fold	1.5 fold	1 fold	8 fold	1 fold	1 fold	1 fold
Nts	6 fold	3 fold	15 fold	1 fold	1 fold	11 fold	2 fold	2 fold	4 fold
Klf4	3 fold	1.4 fold	4 fold	1.8 fold	3 fold	1 fold	2 fold	1.4 fold	3 fold
Tbr1	1 fold	1 fold	1.5 fold	1 fold	1 fold	7 fold	1 fold	1 fold	1 fold
Ttr	97 fold	1.3 fold	33 fold	4 fold	1 fold	29 fold	1 fold	1 fold	1 fold
Egr2	4 fold	2 fold	3 fold	2 fold	2 fold	2 fold	3 fold	1 fold	4 fold
Neurod6	1.5 fold	1 fold	1 fold	1 fold	1 fold	37 fold	1 fold	1 fold	1 fold
Calml4	11 fold	1 fold	32 fold	3 fold	4 fold	1 fold	2 fold	2 fold	1 fold
Cnr1	3 fold	1 fold	1.4 fold	2 fold	3 fold	3 fold	1 fold	1 fold	1 fold

Gene	Foldchange		Groups	
	ko c - ko p	ko c - ko a	ko p - ko a	wt p - ko a
	nicotine induced changes on genotype			
Pttg1	254 fold	2 fold	402 fold	19 fold
Nptx1	1 fold	5 fold	6 fold	6.5 fold
Nts	2 fold	6 fold	3 fold	3 fold
Klf4	2.5 fold	2 fold	1.3 fold	2 fold
Tbr1	1.5 fold	5 fold	7 fold	7 fold
Ttr	3 fold	8 fold	25 fold	32 fold
Egr2	1 fold	4 fold	4 fold	6 fold
Neurod6	1 fold	32 fold	41 fold	39 fold
Calml4	7 fold	1 fold	6 fold	1.3 fold
Cnr1	1.4 fold	1.4 fold	1 fold	3 fold

NCBI	symbol	ko controls - ko aversion		FC	neg. (1 durch x)	
		G_2A-G_2C logFC	G_2A-G_2C FDR		falls Wert kleiner 1	
NM_001083341_at	Mboat2	0,62414557	0,99997714	4,208676742	-0,237604373	
NM_001110239_at	Acp1	0,539843879	0,99997714	3,466122272	-0,288506845	
NM_001122780_at	Klhl25	0,562423164	0,99997714	3,651095258	-0,273890416	
NM_010118_at	Egr2	0,582223811	0,99997714	3,821411542	-0,261683409	
NM_010234_at	Fos	0,569549013	0,99997714	3,711496138	-0,269433124	
NM_010243_at	Fut9	0,61860565	0,99997714	4,155331249	-0,240654701	
NM_020002_at	Rec8	0,852997653	0,99997714	7,128491778	-0,140282129	
NM_020008_at	Clec7a	0,653945381	0,99997714	4,507600112	-0,221847541	
NM_021330_at	Acp1	0,539843879	0,99997714	3,466122272	-0,288506845	**INCREASE**
NM_025727_at	Klhl10	0,547171177	0,99997714	3,525097853	-0,283680068	
NM_026037_at	Mboat2	0,62414557	0,99997714	4,208676742	-0,237604373	
NM_026153_at	Ankrd33b	0,720641669	0,99997714	5,255834351	-0,190264748	
NM_029652_at	Klhl25	0,562423164	0,99997714	3,651095258	-0,273890416	
NM_053128_at	Pcdhb3	0,575511886	0,99997714	3,762806504	-0,265759081	
NM_053230_at	V1rb9	0,544118765	0,99997714	3,500408784	-0,285680919	
NR_003634_at	Rps4y2	0,639875394	0,99997714	4,363906069	-0,229152503	
NM_007726_at	Cnr1	0,157335496	0,99997714	1,436598789	-0,696088572	**CB1**
NM_001139509_at	Nr4a2	-0,958788125	0,99997714	0,109954213	-9,094694704	
NM_008079_at	Galc	-0,702638407	0,99997714	0,198317753	-5,042412921	
NM_008939_at	Prss12	-0,78561627	0,99997714	0,16382634	-6,104024527	
NM_009096_at	Rps6	-0,824043406	0,99997714	0,149953496	-6,668734172	
NM_009114_at	S100a9	-0,76633551	0,99997714	0,171263372	-5,838960133	
NM_009717_at	Neurod6	-1,509153351	0,99997714	0,030963258	-32,29634316	
NM_010478_at	Hspa1b	-0,830304762	0,99997714	0,14780708	-6,765575767	
NM_010930_at	Nov	-1,729608645	0,99997714	0,018637659	-53,65480799	**DECREASE**
NM_013613_at	Nr4a2	-0,958788125	0,99997714	0,109954213	-9,094694704	
NM_013697_at	Ttr	-0,903777952	0,99997714	0,124802145	-8,012682827	
NM_015753_at	Zeb2	-0,92455467	0,99997714	0,118972155	-8,405328087	
NM_024435_at	Nts	-0,757113216	0,99997714	0,174939058	-5,716276346	
NM_031161_at	Cck	-1,245259951	0,99997714	0,056851254	-17,5897615	
NM_153155_at	C1ql3	-0,708783331	0,99997714	0,195531472	-5,114266217	
NM_153163_at	Cadps2	-0,844413137	0,99997714	0,143082613	-6,988969368	

NCBI	symbol	ko controls - ko preferences		FC	neg. (1 durch x) falls Wert kleiner 1	
		G_2A-G_2B logFC	G_2A-G_2B FDR			
NM_001122780_at	Klhl25	0,588000284	0,999869559	3,872578982	-0,25822585	
NM_009909_at	Cxcr2	0,671614823	0,999869559	4,694775429	-0,213002734	
NM_010210_at	Fhit	0,623419427	0,999869559	4,201645689	-0,238001982	
NM_010243_at	Fut9	0,612282861	0,999869559	4,095273027	-0,244183964	
NM_010277_at	Gfap	0,621130358	0,999869559	4,179558016	-0,239259749	
NM_010701_at	Lect1	0,51624194	0,999869559	3,282781218	-0,304619752	
NM_011337_at	Ccl3	0,516893616	0,999869559	3,287710858	-0,304163001	
NM_017372_at	Lyz2	0,599662606	0,999869559	3,977980098	-0,251383862	**INCREASE**
NM_020008_at	Clec7a	0,622306906	0,999869559	4,190896216	-0,238612447	
NM_022987_at	Zic5	0,592292106	0,999869559	3,911038634	-0,255686556	
NM_026153_at	Ankrd33b	0,653864909	0,999869559	4,50676496	-0,221888652	
NM_027998_at	Cldn23	0,566333066	0,999869559	3,684114048	-0,27143568	
NM_029652_at	Klhl25	0,588000284	0,999869559	3,872578982	-0,25822585	
NM_053230_at	V1rb9	0,575684094	0,999869559	3,764298841	-0,265653723	
NM_152839_at	Igj	0,508603799	0,999869559	3,225550153	-0,310024632	
NM_007726_at	Cnr1	0,144680491	0,999869559	1,395341435	-0,716670468	**CB1**
AFFX-r2-Bs-thr-5_s_at		-0,672151984	0,999869559	0,212739442	-4,700585797	
AFFX-ThrX-5_at		-0,660683114	0,999869559	0,218432314	-4,578077222	
NM_001102468_at	Calml4	-0,829573825	0,999869559	0,148056056	-6,754198577	
NM_001131054_at	Pttg1	-2,40408384	0,999869559	0,003943812	-253,5618081	
NM_008856_at	Prkch	-0,641949656	0,999869559	0,228060643	-4,384798657	
NM_009096_at	Rps6	-0,802455477	0,999869559	0,157595758	-6,345348464	
NM_010053_at	Dlx1	-0,556196527	0,999869559	0,277845568	-3,599121658	
NM_011643_at	Trpc1	-0,560426451	0,999869559	0,275152554	-3,634347506	**DECREASE**
NM_013917_at	Pttg1	-1,530665212	0,999869559	0,029466923	-33,93635639	
NM_015753_at	Zeb2	-0,878029855	0,999869559	0,13242505	-7,551441373	
NM_022996_at	Ndfip1	-0,686900921	0,999869559	0,205635968	-4,862962505	
NM_025730_at	Lrrk2	-0,591733519	0,999869559	0,25601563	-3,906011513	
NM_053181_at	Pdxdc1	-0,555717112	0,999869559	0,278152449	-3,595150802	
NM_134052_at	Adi1	-0,709916244	0,999869559	0,195022067	-5,127624855	
NM_138304_at	Calml4	-0,829573825	0,999869559	0,148056056	-6,754198577	

NCBI	symbol	ko preferences - ko aversion		FC	neg. (1 durch x) falls Wert kleiner 1	
		G_2B-G_2C logFC	G_2B-G_2C FDR			
AFFX-r2-Bs-thr-5_s_at		0,66590019	0,999625419	4,633404221	-0,215824036	
AFFX-ThrX-5_at		0,617309889	0,999625419	4,14295188	-0,241373791	
NM_001102468_at	Calml4	0,749969732	0,999625419	5,623021344	-0,177840335	
NM_001109752_at	Dlg4	0,626752145	0,999625419	4,234012589	-0,236182576	
NM_001131054_at	Pttg1	2,605006658	0,999625419	402,7232083	-0,002483095	
NM_001162417_at	Myef2	0,735519922	0,999625419	5,43901081	-0,183856961	
NM_007864_at	Dlg4	0,626752145	0,999625419	4,234012589	-0,236182576	
NM_008856_at	Prkch	0,606997484	0,999625419	4,045735479	-0,247173846	**INCREASE**
NM_010234_at	Fos	0,583579036	0,999625419	3,833354962	-0,260868093	
NM_013917_at	Pttg1	1,830854712	0,999625419	67,74148487	-0,014762003	
NM_019688_at	Rapgef4	0,573647936	0,999625419	3,746691508	-0,266902145	
NM_021716_at	Fign	0,561164466	0,999625419	3,640528757	-0,274685373	
NM_138304_at	Calml4	0,749969732	0,999625419	5,623021344	-0,177840335	
XM_001479366_at	LOC100048025	0,553332101	0,999625419	3,575461459	-0,279684178	
XM_001480960_at	LOC100044139	0,586305492	0,999625419	3,857496068	-0,25923552	
NM_007726_at	Cnr1	0,012655006	0,999625419	1,029567929	-0,971281225	**CB1**
NM_001139509_at	Nr4a2	-0,824122062	0,999625419	0,14992634	-6,66994207	
NM_008079_at	Galc	-0,799288571	0,999625419	0,158749157	-6,299246031	
NM_008730_at	Nptx1	-0,771434478	0,999625419	0,169264359	-5,907918261	
NM_008939_at	Prss12	-0,782592805	0,999625419	0,164970844	-6,061677195	
NM_009114_at	S100a9	-0,711944918	0,999625419	0,194113206	-5,151633018	
NM_009322_at	Tbr1	-0,870137576	0,999625419	0,134853563	-7,415451111	
NM_009717_at	Neurod6	-1,615925228	0,999625419	0,024214459	-41,29763942	
NM_009909_at	Cxcr2	-0,720164076	0,999625419	0,190474097	-5,250057694	**DECREASE**
NM_010217_at	Ctgf	-0,785313306	0,999625419	0,163940666	-6,099767842	
NM_010478_at	Hspa1b	-0,821197781	0,999625419	0,150939261	-6,625181508	
NM_010930_at	Nov	-1,669239446	0,999625419	0,021417095	-46,69167415	
NM_013613_at	Nr4a2	-0,824122062	0,999625419	0,14992634	-6,66994207	
NM_013697_at	Ttr	-1,409946003	0,999625419	0,038909352	-25,70076218	
NM_031161_at	Cck	-1,139207275	0,999625419	0,072575949	-13,77866924	
NM_153163_at	Cadps2	-0,787256375	0,999625419	0,16320882	-6,127119843	

NCBI	symbol	ko untreated - ko controls		FC	neg. (1 durch x) falls Wert kleiner 1	
		G_2-G_2A logFC	G_2-G_2A FDR			
NM_001039543_at	Mlf1	1,255318391	0,999646032	18,00190191	-0,055549686	
NM_001102468_at	Caml4	1,510654082	0,999646032	32,40813819	-0,030856447	
NM_001131054_at	Pttg1	2,438273858	0,999646032	274,3303503	-0,00364524	
NM_008489_at	Lbp	0,98351466	0,987527713	9,62752511	-0,103868854	
NM_008491_at	Lcn2	1,136108895	0,999646032	13,68071813	-0,073095578	
NM_009096_at	Rps6	1,500914451	0,999646032	31,68943171	-0,031556262	
NM_009114_at	S100a9	1,054367381	0,999646032	11,33358695	-0,08823332	**INCREASE**
NM_010053_at	Dlx1	0,989923252	0,999646032	9,7706454	-0,102347384	
NM_010801_at	Mlf1	1,255318391	0,999646032	18,00190191	-0,055549686	
NM_013697_at	Ttr	1,529006747	0,999646032	33,80700883	-0,029579665	
NM_013917_at	Pttg1	1,588919687	0,999646032	38,80785929	-0,025767976	
NM_019394_at	Mia1	1,293140927	0,999646032	19,63997484	-0,050916562	
NM_024435_at	Nts	1,172562524	0,999646032	14,87861561	-0,067210554	
NM_138304_at	Caml4	1,510654082	0,999646032	32,40813819	-0,030856447	
NM_144841_at	Otx2	1,060669	0,999646032	11,49923634	-0,086962296	
NM_007726_at	Cnr1	-0,159836141	0,999646032	0,692092048	-1,44489451	**CB1**
NM_001161775_at	Myh11	-0,602326399	0,738615728	0,24984669	-4,002454458	
NM_001168655_at	Socs2	-0,616931412	0,243343642	0,241584234	-4,139342971	
NM_001168656_at	Socs2	-0,616931412	0,243343642	0,241584234	-4,139342971	
NM_001168657_at	Socs2	-0,616931412	0,243343642	0,241584234	-4,139342971	
NM_007706_at	Socs2	-0,616931412	0,243343642	0,241584234	-4,139342971	
NM_008452_at	Klf2	-1,123483713	0,227639316	0,075251695	-13,2887372	
NM_008597_at	Mgp	-0,60901774	0,738615728	0,246026711	-4,064599318	**DECREASE**
NM_008963_at	Ptgds	-0,677904445	0,82883542	0,209940175	-4,763261723	
NM_009263_at	Spp1	-0,687772933	0,999646032	0,205223489	-4,872736568	
NM_010217_at	Ctgf	-0,633749568	0,82883542	0,232407657	-4,302784228	
NM_010234_at	Fos	-1,359875836	0,990014242	0,043664065	-22,90212792	
NM_013607_at	Myh11	-0,602326399	0,738615728	0,24984669	-4,002454458	
NM_013642_at	Dusp1	-0,658939612	0,999646032	0,219310986	-4,559735091	
NM_026153_at	Ankrd33b	-0,92681721	0,990181644	0,118353959	-8,44923151	
NM_026878_at	Rasl11b	-0,830714493	0,601583727	0,147667699	-6,771961697	

NCBI	symbol	wt aversion - ko aversion		FC	neg. (1 durch x) falls Wert kleiner 1	
		G_1C-G_2C logFC	G_1C-G_2C FDR			
AFFX-18SRNAMur/X00686_5_at		0,561722136	0,844646963	3,6452065	-0,274332881	
NM_001109752_at	Dlg4	0,52589639	0,862867268	3,356575266	-0,29792271	
NM_001122780_at	Klhl25	0,591183807	0,602645901	3,901070572	-0,25633989	
NM_001131054_at	Pttg1	3,977960205	0,426954166	9505,176924	-0,000105206	
NM_001161712_at	Gcat	0,530011502	0,657953292	3,388531303	-0,295113107	
NM_001162417_at	Myef2	0,542798867	0,726481264	3,48978657	-0,286550475	
NM_007524_at	Nkx3-2	0,518646904	0,657953292	3,301010487	-0,302937541	
NM_007864_at	Dlg4	0,52589639	0,862867268	3,356575266	-0,29792271	**INCREASE**
NM_008169_at	Grin1	0,548061443	0,755688898	3,532331409	-0,283099145	
NM_010517_at	Igfbp4	0,543481435	0,657953292	3,495275679	-0,286100466	
NM_013917_at	Pttg1	2,897126051	0,417271613	789,0891121	-0,001267284	
NM_029652_at	Klhl25	0,591183807	0,602645901	3,901070572	-0,25633989	
NM_030715_at	Polh	0,544807833	0,343506707	3,505967073	-0,285228007	
NM_053095_at	Il24	0,514286005	0,602645901	3,268029777	-0,305994764	
XM_909511_at	LOC634699	0,578841315	0,411766961	3,79176414	-0,263729484	
NM_007726_at	Cnr1	0,454977204	0,657953292	2,850868622	-0,350770285	**CB1**
NM_001111119_at	Ccnb1ip1	-1,118980842	0,802484915	0,076035982	-13,15166815	
NM_001139509_at	Nr4a2	-0,767626819	0,587946589	0,170754902	-5,856347235	
NM_008730_at	Nptx1	-0,917875212	0,401903303	0,120816093	-8,277043011	
NM_009263_at	Spp1	-1,029629826	0,772149549	0,093405011	-10,70606377	
NM_009322_at	Tbr1	-0,825186973	0,654503927	0,149559163	-6,686317158	
NM_009717_at	Neurod6	-1,573550236	0,609402836	0,026696219	-37,45848738	
NM_010217_at	Ctgf	-1,014754299	0,343506707	0,096659757	-10,34556703	
NM_010930_at	Nov	-1,717679657	0,52445325	0,019156684	-52,20110028	**DECREASE**
NM_013613_at	Nr4a2	-0,767626819	0,587946589	0,170754902	-5,856347235	
NM_013697_at	Ttr	-1,459664592	0,81458257	0,034700474	-28,8180501	
NM_024254_at	2410042D21Rik	-0,802922005	0,714785941	0,157426556	-6,352168429	
NM_024435_at	Nts	-1,058091805	0,751034634	0,087479883	-11,43119952	
NM_031161_at	Cck	-1,158513265	0,654503927	0,06942034	-14,40500008	
NM_153163_at	Cadps2	-1,042446191	0,488400665	0,090688832	-11,02671605	
XM_907202_at	Ccnb1ip1	-1,118980842	0,802484915	0,076035982	-13,15166815	

NCBI	symbol	wt controls - ko controls		FC	neg. (1 durch x) falls Wert kleiner 1	
		G_1A-G_2A logFC	G_1A-G_2A FDR			
NM_001131054_at	Pttg1	4,03165215	0,999953634	10756,03359	-9,29711E-05	
NM_001167763_at	Ift122	0,653542747	0,999953634	4,503423057	-0,222053311	
NM_009096_at	Rps6	0,931822571	0,999953634	8,547174507	-0,116997728	
NM_010478_at	Hspa1b	2,068316532	0,999953634	117,0352082	-0,008544437	
NM_010479_at	Hspa1a	1,478882403	0,999953634	30,12190282	-0,033198434	
NM_011254_at	Rbp1	0,814787501	0,999953634	6,52811056	-0,15318368	
NM_013917_at	Pttg1	2,870430459	0,999953634	742,0453689	-0,001347626	
NM_015753_at	Zeb2	1,097481407	0,999953634	12,51645686	-0,079894815	**INCREASE**
NM_026038_at	2810055F11Rik	0,563864201	0,999953634	3,663230117	-0,272983124	
NM_029803_at	Ifi27l2a	0,805002395	0,999953634	6,38267006	-0,156674243	
NM_031177_at	Ift122	0,653542747	0,999953634	4,503423057	-0,222053311	
NM_145451_at	Gpx6	0,663376632	0,999953634	4,606558937	-0,217081777	
NM_145962_at	Pank3	0,767414217	0,999953634	5,853481054	-0,170838513	
NM_170779_at	Wwc1	0,855362023	0,999953634	7,167406282	-0,139520485	
XM_001473622_at	Rbp1	0,814787501	0,999953634	6,52811056	-0,15318368	
NM_007726_at	Cnr1	0,319415601	0,999953634	2,086486605	-0,479274584	**CB1**
NM_001037127_at	Musk	-0,513180312	0,999953634	0,306774804	-3,259720112	
NM_001037128_at	Musk	-0,513180312	0,999953634	0,306774804	-3,259720112	
NM_001037129_at	Musk	-0,513180312	0,999953634	0,306774804	-3,259720112	
NM_001037130_at	Musk	-0,513180312	0,999953634	0,306774804	-3,259720112	
NM_007428_at	Agt	-0,633778995	0,999953634	0,23239191	-4,303075786	
NM_008407_at	Itih3	-0,714454785	0,999953634	0,192994626	-5,18149144	
NM_008449_at	Kif5c	-0,549019549	0,999953634	0,282475282	-3,54013276	
NM_008963_at	Ptgds	-0,8457054	0,999953634	0,142657497	-7,009796335	**DECREASE**
NM_009242_at	Sparc	-0,536024434	0,999953634	0,291055336	-3,435772775	
NM_009263_at	Spp1	-0,628649171	0,999953634	0,235153165	-4,252547478	
NM_010234_at	Fos	-0,672384881	0,999953634	0,212625388	-4,703107233	
NM_010944_at	Musk	-0,513180312	0,999953634	0,306774804	-3,259720112	
NM_013697_at	Ttr	-0,55586109	0,999953634	0,278060251	-3,59634287	
NM_026153_at	Ankrd33b	-0,782261272	0,999953634	0,165096828	-6,05705158	
NM_027998_at	Cldn23	-0,521341013	0,999953634	0,301064111	-3,321551672	
XM_001476727_at	LOC100046740	-0,519230091	0,999953634	0,302531018	-3,305446186	

NCBI	symbol	wt controls - wt aversion		FC	neg. (1 durch x) falls Wert kleiner 1	
		G_1A-G_1C logFC	G_1A-G_1C FDR			
NM_001025431_at	Btbd3	0,574843065	0,606660494	3,757016177	-0,26616867	
NM_001111119_at	Ccnb1ip1	1,110825572	0,675575037	12,90700778	-0,077477291	
NM_001145799_at	Ctla2a	0,680666509	0,606660494	4,793652068	-0,208609216	
NM_007796_at	Ctla2a	0,680666509	0,606660494	4,793652068	-0,208609216	
NM_009263_at	Spp1	0,631044161	0,789628976	4,276063649	-0,233859943	
NM_010277_at	Gfap	0,859915691	0,617217905	7,2429534	-0,138065226	
NM_010387_at	H2-DMb1	0,601365776	0,606660494	3,993611148	-0,250399942	
NM_010478_at	Hspa1b	0,840742692	0,744453868	6,930150912	-0,144297002	**INCREASE**
NM_010479_at	Hspa1a	0,701419402	0,707031167	5,028279405	-0,198875186	
NM_021272_at	Fabp7	0,719965434	0,606660494	5,247656919	-0,190561238	
NM_024254_at	2410042D21Rik	0,581617018	0,653892545	3,816076023	-0,262049287	
NM_026038_at	2810055F11Rik	0,863908127	0,606660494	7,309844307	-0,136801819	
NM_145534_at	Btbd3	0,574843065	0,606660494	3,757016177	-0,26616867	
NM_178254_at	Tcfl5	0,571597638	0,710130777	3,729045118	-0,268165165	
XM_907202_at	Ccnb1ip1	1,110825572	0,675575037	12,90700778	-0,077477291	
NM_007726_at	Cnr1	0,021773893	0,97574448	1,051414333	-0,951099836	**CB1**
AFFX-18SRNAMur/X00686_5_at		-0,453411847	0,798857021	0,352036872	-2,84061154	
AFFX-r2-Bs-thr-5_s_at		-0,456347713	0,853675408	0,349665099	-2,859879357	
NM_001122667_at	Mkl2	-0,461590697	0,606660494	0,345469175	-2,894614258	
NM_001165965_at	Fndc7	-0,511282473	0,315728775	0,308118324	-3,245506423	
NM_001171002_at	Degs2	-0,795233916	0,606660494	0,16023821	-6,240708767	
NM_007689_at	Chad	-0,47094302	0,606660494	0,338109194	-2,957624397	
NM_008079_at	Galc	-0,488914339	0,617217905	0,324403597	-3,082579877	
NM_009040_at	Rdh16	-0,453423478	0,606660494	0,352027444	-2,840687616	**DECREASE**
NM_010234_at	Fos	-0,494719637	0,795546521	0,320096085	-3,124061948	
NM_026161_at	C1qtnf4	-0,461952255	0,606660494	0,345181685	-2,89702508	
NM_080459_at	Strc	-0,46232986	0,606660494	0,344881692	-2,899545045	
NM_177091_at	Fndc7	-0,511282473	0,315728775	0,308118324	-3,245506423	
XM_001004899_at	LOC677567	-0,542974755	0,606660494	0,286434447	-3,49120021	
XM_001471730_at	LOC100044249	-0,468124079	0,606660494	0,340310948	-2,938489066	
XM_001474590_at	LOC100045607	-0,455866671	0,644690808	0,350052617	-2,856713394	

NCBI	symbol	wt controls - wt preferences		FC	neg. (1 durch x)	
		G_1A-G_1B logFC	G_1A-G_1B FDR		falls Wert kleiner 1	
NM_001039071_at	Ldb3	0,496634102	0,999924502	3,137863894	-0,318688137	
NM_001039072_at	Ldb3	0,496634102	0,999924502	3,137863894	-0,318688137	
NM_001039073_at	Ldb3	0,496634102	0,999924502	3,137863894	-0,318688137	
NM_001039074_at	Ldb3	0,496634102	0,999924502	3,137863894	-0,318688137	
NM_001131054_at	Pttg1	2,961552553	0,999924502	915,2770082	-0,001092565	
NM_010478_at	Hspa1b	0,504692324	0,999924502	3,196629654	-0,312829482	
NM_011918_at	Ldb3	0,496634102	0,999924502	3,137863894	-0,318688137	
NM_013917_at	Pttg1	2,268006607	0,999924502	185,3559822	-0,005395024	**INCREASE**
NM_145451_at	Gpx6	0,849126067	0,999924502	7,065226139	-0,141538286	
NM_145962_at	Pank3	0,464018327	0,999924502	2,910839951	-0,34354345	
NM_170779_at	Wwc1	0,513994548	0,999924502	3,265837323	-0,306200187	
NM_175656_at	Hist1h4i	0,840325351	0,999924502	6,923494491	-0,144435733	
NM_198885_at	Scx	0,603018899	0,999924502	4,008841623	-0,249448617	
NR_028275_at	Snord14e	0,574828381	0,999924502	3,75688915	-0,26617767	
XM_001472216_at	LOC100044411	0,475256046	0,999924502	2,987143223	-0,334768013	
NM_007726_at	Cnr1	0,037280225	0,999924502	1,089632943	-0,917740241	**CB1**
NM_001081237_at	Klhdc5	-0,391757276	0,999924502	0,405735235	-2,464661471	
NM_001171002_at	Degs2	-0,50365981	0,999924502	0,313574104	-3,18903885	
NM_008483_at	Lamb2	-0,382490507	0,999924502	0,414485644	-2,412628796	
NM_008537_at	Amacr	-0,413534053	0,999924502	0,385892153	-2,591397605	
NM_008963_at	Ptgds	-0,426988573	0,999924502	0,374120432	-2,672936078	
NM_009312_at	Tac2	-0,441035141	0,999924502	0,362213689	-2,760801238	
NM_010052_at	Dlk1	-0,424565592	0,999924502	0,376213528	-2,65806497	
NM_010118_at	Egr2	-0,467035183	0,999924502	0,341165272	-2,931130692	**DECREASE**
NM_021565_at	Midn	-0,406271469	0,999924502	0,392399577	-2,548422724	
NM_022889_at	Pes1	-0,553222567	0,999924502	0,279754727	-3,574559801	
NM_026153_at	Ankrd33b	-0,467499949	0,999924502	0,340800365	-2,934269159	
NM_134052_at	Adi1	-0,573506896	0,999924502	0,266988837	-3,745474943	
NM_172893_at	Parp12	-0,406498719	0,999924502	0,392194303	-2,549756567	
NR_033611_at		-0,463646684	0,999924502	0,34383756	-2,908350096	
XM_001475816_at	LOC100046254	-0,380632156	0,999924502	0,416263033	-2,402327186	

NCBI	symbol	wt preference - ko aversion		Fold Change	neg. (1 durch x) falls Wert kleiner 1	
		G_1B-G_2C logFC	G_1B-G_2C FDR			
NM_001131054_at	Pttg1	1,271022415	0,999699964	18,66476022	-0,0535769	
NM_001159321_at	Grid2ip	0,569248245	0,999699964	3,708926651	-0,269619783	
NM_009780_at	C4b	0,585922146	0,999699964	3,854092606	-0,259464445	
NM_010118_at	Egr2	0,80676331	0,999699964	6,40860213	-0,156040269	
NM_010478_at	Hspa1b	0,733319446	0,999699964	5,411522222	-0,184790889	
NM_010479_at	Hspa1a	0,767006774	0,999699964	5,84799205	-0,170998864	
NM_011254_at	Rbp1	0,59913732	0,999699964	3,97317158	-0,251688098	**INCREASE**
NM_013917_at	Pttg1	0,902613352	0,999699964	7,991224878	-0,125137262	
NM_023653_at	Wnt2	0,633978602	0,999699964	4,305053985	-0,232285124	
NM_028810_at	Rnd3	0,660391864	0,999699964	4,575008065	-0,21857885	
XM_001473622_at	Rbp1	0,59913732	0,999699964	3,97317158	-0,251688098	
XM_001479366_at	LOC100048025	0,646174786	0,999699964	4,427665324	-0,225852662	
XM_972987_at	LOC675521	0,585922146	0,999699964	3,854092606	-0,259464445	
XM_973028_at	LOC675521	0,585922146	0,999699964	3,854092606	-0,259464445	
XM_973068_at	LOC675521	0,585922146	0,999699964	3,854092606	-0,259464445	
NM_007726_at	Cnr1	0,439470872	0,999699964	2,750875097	-0,363520685	**CB1**
NM_001139509_at	Nr4a2	-0,680765894	0,999699964	0,208561483	-4,794749183	
NM_008613_at	Mns1	-0,725275881	0,999699964	0,18824529	-5,312217901	
NM_008730_at	Nptx1	-0,816031053	0,999699964	0,152745684	-6,546829833	
NM_008939_at	Prss12	-0,826336964	0,999699964	0,149163662	-6,704045672	
NM_009114_at	S100a9	-0,886994228	0,999699964	0,129719651	-7,708932233	
NM_009322_at	Tbr1	-0,833742272	0,999699964	0,146641781	-6,819338873	
NM_009717_at	Neurod6	-1,588001604	0,999699964	0,025822507	-38,72590749	
NM_010217_at	Ctgf	-0,789211787	0,999699964	0,162475624	-6,15476941	**DECREASE**
NM_010930_at	Nov	-1,77376802	0,999699964	0,016835731	-59,39747996	
NM_013613_at	Nr4a2	-0,680765894	0,999699964	0,208561483	-4,794749183	
NM_013697_at	Ttr	-1,503313505	0,999699964	0,031382425	-31,86496937	
NM_031161_at	Cck	-1,183705389	0,999699964	0,065508041	-15,26530158	
NM_145451_at	Gpx6	-0,704324224	0,999699964	0,197549428	-5,062024277	
NM_153155_at	C1ql3	-0,728429879	0,999699964	0,186883139	-5,350937512	
NM_153163_at	Cadps2	-0,774867186	0,999699964	0,16793175	-5,954800085	

NCBI	symbol	wt preferences - ko preferences		FC	neg. (1 durch x)	
		G_1B-G_2B logFC	G_1B-G_2B FDR		falls Wert kleiner 1	
NM_001159321_at	Grid2ip	0,493057772	0,999979901	3,112130301	-0,321323307	
NM_008331_at	Ifit1	0,545120366	0,999979901	3,508490993	-0,285022821	
NM_009909_at	Cxcr2	0,557055932	0,999979901	3,606250843	-0,277296296	
NM_010478_at	Hspa1b	1,554517227	0,999979901	35,85231694	-0,0278922	
NM_010479_at	Hspa1a	1,243963872	0,999979901	17,53734606	-0,05702117	
NM_010637_at	Klf4	0,460938656	0,999979901	2,890271603	-0,345988245	
NM_011254_at	Rbp1	0,630820312	0,999979901	4,273860199	-0,233980513	
NM_011909_at	Usp18	0,508985095	0,999979901	3,228383322	-0,30975256	**INCREASE**
NM_012011_at	Eif2s3y	0,497108051	0,999979901	3,141290139	-0,31834054	
NM_022987_at	Zic5	0,474400069	0,999979901	2,981261482	-0,335428478	
NM_029803_at	Ifi27l2a	0,542525552	0,999979901	3,48759103	-0,286730867	
XM_001473622_at	Rbp1	0,630820312	0,999979901	4,273860199	-0,233980513	
XM_001475816_at	LOC100046254	0,516713272	0,999979901	3,286345895	-0,304289333	
XM_001480051_at	LOC100048346	0,508985095	0,999979901	3,228383322	-0,30975256	
XR_034160_at	LOC100047829	0,457435253	0,999979901	2,867049908	-0,34879058	
NM_007726_at	Cnr1	0,426815866	0,999979901	2,671873336	-0,374269239	**CB1**
NM_001102468_at	Calml4	-0,642172334	0,999979901	0,227943738	-4,387047473	
NM_001113331_at	Shc1	-0,495371495	0,999979901	0,319615995	-3,128754556	
NM_001131054_at	Pttg1	-1,333984243	0,999979901	0,046346373	-21,57666124	
NM_001145885_at	Ddx4	-0,585099424	0,999979901	0,259956437	-3,846798376	
NM_007428_at	Agt	-0,488034229	0,999979901	0,325061677	-3,076339267	
NM_008613_at	Mns1	-0,579153788	0,999979901	0,2635398	-3,79449328	
NM_009263_at	Spp1	-0,619858581	0,999979901	0,239961418	-4,167336607	
NM_010029_at	Ddx4	-0,585099424	0,999979901	0,259956437	-3,846798376	**DECREASE**
NM_010234_at	Fos	-0,57476784	0,999979901	0,266214778	-3,756365473	
NM_011368_at	Shc1	-0,495371495	0,999979901	0,319615995	-3,128754556	
NM_013917_at	Pttg1	-0,92824136	0,999979901	0,117966485	-8,476983933	
NM_019688_at	Rapgef4	-0,522555313	0,999979901	0,300223502	-3,330851823	
NM_133435_at	Nmnat1	-0,477855486	0,999979901	0,332770266	-3,00507618	
NM_138304_at	Calml4	-0,642172334	0,999979901	0,227943738	-4,387047473	
NM_175656_at	Hist1h4i	-0,497089392	0,999979901	0,318354218	-3,14115518	

NCBI	symbol	wt preferences - wt aversion		FC	neg. (1 durch x)	
		G_1B-G_1C logFC	G_1B-G_1C FDR		falls Wert kleiner 1	
NM_001042726_at	Pcdh8	0,566925437	0,671793214	3,689142551	-0,271065698	
NM_001111119_at	Ccnb1ip1	0,788002927	0,840802524	6,137661417	-0,162928505	
NM_008537_at	Amacr	0,597005998	0,671793214	3,953720805	-0,252926306	
NM_008731_at	Npy2r	0,56371608	0,671793214	3,661980944	-0,273076244	
NM_010118_at	Egr2	0,551891042	0,671793214	3,563617164	-0,280613757	
NM_010277_at	Gfap	0,617577025	0,794848602	4,145501007	-0,241225367	
NM_010387_at	H2-DMb1	0,750356833	0,475076452	5,628035563	-0,177681891	
NM_021472_at	Rnase4	0,640428286	0,475076452	4,369465212	-0,228860959	**INCREASE**
NM_021543_at	Pcdh8	0,566925437	0,671793214	3,689142551	-0,271065698	
NM_024435_at	Nts	0,565240666	0,84602213	3,674858878	-0,272119293	
NM_026038_at	2810055F11Rik	0,600701695	0,737845954	3,987509172	-0,250783122	
NM_134052_at	Adi1	0,600388075	0,712233322	3,984630684	-0,250964287	
NM_178254_at	Tcfl5	0,576346322	0,800784603	3,770043159	-0,265248953	
NM_201239_at	Rnase4	0,640428286	0,475076452	4,369465212	-0,228860959	
XM_907202_at	Ccnb1ip1	0,788002927	0,840802524	6,137661417	-0,162928505	
NM_007726_at	Cnr1	-0,015506332	0,989183759	0,964925243	-1,036349714	**CB1**
NM_001113331_at	Shc1	-0,624642482	0,475076452	0,237332666	-4,213494991	
NM_001122667_at	Mkl2	-0,471023548	0,671793214	0,338046506	-2,958172858	
NM_001131054_at	Pttg1	-2,70693779	0,671793214	0,001963642	-509,2579178	
NM_001145885_at	Ddx4	-0,582822792	0,685970729	0,261322743	-3,826685687	
NM_010029_at	Ddx4	-0,582822792	0,685970729	0,261322743	-3,826685687	
NM_011105_at	Pkdrej	-0,512025671	0,585568718	0,307591499	-3,251065138	
NM_011368_at	Shc1	-0,624642482	0,475076452	0,237332666	-4,213494991	
NM_013917_at	Pttg1	-1,994512699	0,671793214	0,010127151	-98,74445087	**DECREASE**
NM_026047_at	Rnf219	-0,47622851	0,482595252	0,334019245	-2,993839472	
NM_027495_at	Tmem144	-0,505359962	0,671793214	0,312348941	-3,201547591	
NM_030562_at	Lrfn1	-0,488919146	0,671793214	0,324400006	-3,082613997	
NM_080459_at	Strc	-0,541359818	0,510989024	0,287501544	-3,478242185	
NM_133362_at	Erdr1	-0,5457857	0,927644458	0,284586504	-3,513870079	
NM_145451_at	Gpx6	-0,61015027	0,793256834	0,245385971	-4,075212596	
XR_031725_at	LOC100045480	-0,582411752	0,921863941	0,26157019	-3,823065616	

NCBI	symbol	wt untreated - ko untreated		FC	neg. (1 durch x)	
		G_1-G_2 logFC	G_1-G_2 FDR		falls Wert kleiner 1	
NM_001126318_at	Gm13011	0,562020908	0,8812628	3,647715075	-0,274144219	
NM_001131054_at	Pttg1	0,937292036	0,917559385	8,655497514	-0,115533509	
NM_007726_at	Cnr1	0,483927854	0,850667493	3,047388708	-0,328149802	
NM_010118_at	Egr2	0,613796518	0,850667493	4,109571283	-0,243334385	
NM_010444_at	Nr4a1	0,474938142	0,850667493	2,984957431	-0,335013153	
NM_010478_at	Hspa1b	0,754713117	0,890991708	5,684772863	-0,175908523	
NM_010637_at	Klf4	0,482144342	0,850667493	3,034899695	-0,329500181	
NM_011060_at	Padi3	0,699125273	0,562200749	5,001787916	-0,199928509	**INCREASE**
NM_013616_at	Olfr65	0,545144318	0,850667493	3,508684497	-0,285007102	
NM_013917_at	Pttg1	0,623209426	0,917706424	4,199614495	-0,238117094	
NM_019819_at	Dusp14	0,632227421	0,850667493	4,287729913	-0,233223645	
NM_026268_at	Dusp6	0,520282321	0,850667493	3,313464493	-0,301798918	
NM_028810_at	Rnd3	0,508946849	0,873800216	3,228099028	-0,30977984	
NM_139306_at	Acer2	0,550829718	0,882294635	3,554919068	-0,281300356	
XR_034282_at	Gm13011	0,562020908	0,8812628	3,647715075	-0,274144219	
	Cnr1	0,483927854	0,850667493	3,047388708	-0,328149802	**CB1**
NM_001039543_at	Mlf1	-0,882376283	0,882294635	0,131106347	-7,627395794	
NM_001102468_at	Calml4	-1,034949549	0,882294635	0,092267861	-10,83801004	
NM_008579_at	Meig1	-1,026408904	0,859041334	0,094100319	-10,62695653	
NM_009846_at	Cd24a	-0,886495596	0,882294635	0,129868673	-7,70008636	
NM_010053_at	Dlx1	-0,931521086	0,882294635	0,117078976	-8,541243161	
NM_010801_at	Mlf1	-0,882376283	0,882294635	0,131106347	-7,627395794	
NM_013697_at	Ttr	-1,986766305	0,859041334	0,010309407	-96,99878738	
NM_019394_at	Mia1	-0,978041688	0,882294635	0,10518609	-9,506960467	**DECREASE**
NM_023842_at	Dsp	-0,866126509	0,859041334	0,136104815	-7,347278616	
NM_028567_at	1700094D03Rik	-0,804959738	0,850667493	0,156689633	-6,382043176	
NM_028813_at	Vit	-0,819549955	0,850667493	0,151513051	-6,600091479	
NM_138304_at	Calml4	-1,034949549	0,882294635	0,092267861	-10,83801004	
XM_001481272_at	Dsp	-0,874832058	0,859041334	0,133403721	-7,496042807	
XM_621314_at	Dsp	-0,866126509	0,859041334	0,136104815	-7,347278616	
XM_896350_at	Dsp	-0,866126509	0,859041334	0,136104815	-7,347278616	

NCBI	symbol	wt untreated - wt controls		FC	neg. (1 durch x) falls Wert kleiner 1	
		G_1-G_1A logFC	G_1-G_1A FDR			
NM_001163434_at	Hspa5	0,504887236	0,317885811	3,198064629	-0,312689115	
NM_008079_at	Galc	0,480900677	0,720878697	3,026221253	-0,330445105	
NM_008449_at	Kif5c	0,458327572	0,720878697	2,872946721	-0,348074676	
NM_008491_at	Lcn2	1,37681973	0,791903053	23,81330805	-0,041993326	
NM_009114_at	S100a9	0,516158507	0,916679742	3,282150618	-0,304678278	
NM_009236_at	Sox18	0,670543969	0,36421191	4,683213629	-0,213528589	
NM_009991_at	Cyp11b2	0,485429558	0,571076101	3,057944212	-0,327017084	
NM_019819_at	Dusp14	0,478377006	0,737151573	3,008686976	-0,332370901	**INCREASE**
NM_022310_at	Hspa5	0,504887236	0,317885811	3,198064629	-0,312689115	
NM_023493_at	Cml5	0,471078404	0,691817975	2,95854653	-0,33800381	
NM_024435_at	Nts	0,485312458	0,902566463	3,057119801	-0,327105271	
NM_176962_at	6330416L07Rik	0,464605814	0,688646597	2,914780222	-0,34307904	
XM_001472518_at	LOC100044106	0,634384898	0,711897625	4,309083383	-0,232067916	
XM_001478891_at		0,545542063	0,257690213	3,511899369	-0,2847462	
XM_001478992_at		0,545542063	0,257690213	3,511899369	-0,2847462	
	Cnr1	0,004676112	0,997036911	1,01082532	-0,989290612	**CB1**
NM_001111119_at	Ccnb1ip1	-1,081001115	0,791994248	0,082984864	-12,05039034	
NM_010277_at	Gfap	-0,6836637	0,791994248	0,2071745	-4,826848857	
NM_010398_at	H2-T23	-0,688903442	0,329442706	0,204689968	-4,885437279	
NM_010478_at	Hspa1b	-1,699462312	0,680537798	0,019977341	-50,05671115	
NM_010479_at	Hspa1a	-1,390620868	0,540145731	0,04067983	-24,58220681	
NM_013917_at	Pttg1	-0,658301345	0,908871188	0,219633537	-4,553038734	
NM_015753_at	Zeb2	-0,785399855	0,688646597	0,163907998	-6,100983564	
NM_026878_at	Rasl11b	-0,827756386	0,317885811	0,14867694	-6,725992613	**DECREASE**
NM_029083_at	Ddit4	-0,668205512	0,041885884	0,214681434	-4,658064651	
NM_172543_at	Fam117a	-0,674500853	0,36421191	0,211591953	-4,726077651	
NR_028275_at	Snord14e	-0,944738722	0,691817975	0,113569386	-8,805189804	
XM_001473226_at	C920025E04Rik	-0,688903442	0,329442706	0,204689968	-4,885437279	
XM_907202_at	Ccnb1ip1	-1,081001115	0,791994248	0,082984864	-12,05039034	
XM_975970_at	C920025E04Rik	-0,688903442	0,329442706	0,204689968	-4,885437279	
XM_992574_at	C920025E04Rik	-0,688903442	0,329442706	0,204689968	-4,885437279	

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Circuit Specific Functions of Cannabinoid CB1 Receptor in the Balance of Investigatory Drive and Exploration

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Abstract

Well balanced novelty seeking and exploration are fundamental behaviours for survival and are found to be dysfunctional in several psychiatric disorders. Recent studies suggest that the endocannabinoid (eCB) system is an important control system for investigatory drive. Pharmacological treatment of rodents with cannabinergic drugs results in altered social and object investigation. Interestingly, contradictory results have been obtained, depending on the treatment, drug concentration and experimental conditions. The cannabinoid type 1 (CB1) receptor, a central component of the eCB system, is predominantly found at the synapses of two opposing neuronal populations, i.e. on inhibitory GABAergic and excitatory glutamatergic neurons. In the present study, using different transgenic mouse lines, we aimed at investigating the impact of CB1 receptor inactivation in glutamatergic or GABAergic neurons on investigatory behaviour. We evaluated animate (interaction partner) and inanimate (object) exploratory behaviour in three different paradigms. We show that exploration was increased when CB1 receptor was deleted from cortical and striatal GABAergic neurons. No effect was observed when CB1 receptor was deleted specifically from dopamine receptor D1-expressing striatal GABAergic medium spiny neurons. In contrast, deletion of CB1 receptor from cortical glutamatergic neurons resulted in a decreased exploration. Thus, our results indicate that exploratory behaviour is accurately balanced in both, the social and non-social context, by the eCB system via CB1 receptor activation on cortical glutamatergic and GABAergic neurons. In addition, the results could explain the contradictory findings of previous pharmacological studies and could further suggest a possibility to readjust an imbalance in exploratory behaviour observed in psychiatric disorders.

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Introduction

Adequate novelty seeking and exploration are fundamental behaviours for survival. Dysfunctional exploratory profiles have been found in several distinct neuronal disorders, such as attention deficit disorder and schizophrenia-like diseases, expressed by modulated social behaviour and novelty seeking [1–5]. Thus, identifying control mechanisms of exploratory behaviour might allow new treatment strategies. Two recent studies indicated that the endocannabinoid (eCB) system might be important for a balanced response to novel situations [6,7], but these studies elucidated only some aspects on the function of the eCB system in exploratory behaviour. Lafenêtre et al. [6] concentrated on object recognition with repeated exposures to a novel object and food pellet, thus, strongly reducing the novelty factor every day. Jacob et al. [7] performed multiple behavioural paradigms, including also social interaction studies. However, the latter study was only performed with animals lacking the cannabinoid type 1 (CB1) receptor completely or specifically in cortical glutamatergic neurons. To this end, the present study aimed at further detailing CB1 receptor functions in investigatory drive and exploration behaviour.

One important factor in exploratory behaviour is how a respective situation is evaluated. Brain regions involved in these evaluation processes, such as amygdala, hippocampus, and prefrontal cortex, show high levels of CB1 receptor mRNA and protein [8,9]. These cortical areas possess two major neuronal subpopulations expressing the CB1 receptor; GABAergic interneurons (with high CB1 receptor levels) and glutamatergic neurons (with low CB1 receptor levels) [8,10–12]. The two neuronal populations represent the two major opposing players regarding the excitation state of the brain, namely GABAergic interneurons being inhibitory and glutamatergic neurons being excitatory. The endogenous ligands of CB1 receptor, the so-called endocannabinoids, are synthesized at the postsynapse and travel retrogradely to the CB1 receptor located at the presynapse [13]. Activated CB1 receptor then suppresses neurotransmitter release. Therefore, a functional eCB system may have a protective role to prevent an imbalance of neuronal activity and inadequate behavioural responses. In accordance with this notion, it was shown that the inactivation of the CB1 receptor gene from glutamatergic neurons leads to an increased vulnerability to kainic acid-induced seizures [10]. Furthermore, other behavioural studies indicated a bidirectional role of the eCB system in anxiety response based on CB1 receptor located on these two neuronal subpopulations [6,7,14].

Anxiety plays a critical role in exploratory and investigatory behaviour, and several pharmacological studies have shown the

importance of the eCB system in social behaviour [15–19]. The results depended strongly on the treatment and experimental conditions, and they seemed to be contradictory at first sight. The acute and chronic administration of CB1 receptor agonists (Δ^9-THC or WIN55,212-2), especially during adolescence, led to a decreased social interaction in rats. Opposing to this, treatment of adolescent rats with URB597, an inhibitor of anandamide degradation, or VDM11, a putative anandamide reuptake inhibitor, resulted in increased social play behaviour [17–19]. These latter findings are supported by studies with transgenic mice lacking the CB1 receptor ubiquitously or specifically in cortical glutamatergic neurons, where a decrease in object exploration and social interaction was shown, depending on the behavioural context [7,20]. Altogether, these data suggest that strong systemic activation of the eCB system has anti-social effects, whereas on-demand enhancement of anandamide signalling and subsequent activation of CB1 receptor has a pro-social effect.

By using several conditional CB1 receptor knock-out mice, we aimed at investigating whether CB1 receptor on different neuronal cell types might explain the contradictory findings in social interaction and object exploration mentioned above. In order to address this question, we applied different behavioural paradigms to analyze inanimate (object) exploration and animate (interaction partner) exploration. Evaluating the results, we could detect a decreased exploratory drive in mice lacking CB1 receptor in cortical glutamatergic neurons. Mice lacking CB1 receptor in GABAergic neurons, including the striatum, displayed opposite results, namely, an increased exploratory drive. No changes in exploration were observed for mice lacking CB1 receptor specifically in striatal dopamine receptor D1-positive GABAergic medium spiny neurons. Thus, we hypothesize that cortical GABAergic interneurons are important for the increased exploratory drive. Altogether, our results suggest that exploratory behaviour (animate and inanimate) is balanced by the eCB system via CB1 receptor activation on the two opposing neuronal subpopulations.

Materials and Methods

Animals

This study was performed on adult (5–7 months old) male mutant mice and their respective wild-type littermates. Animals were housed in a temperature- and humidity-controlled room (22°C±1; 50%±1) with a 12 h light-dark cycle (lights on at 1 am) and had access to food and water *ad libitum*. The experimental protocols were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethical Committee on animal care and use of Rhineland-Palatinate, Germany. Generation, breeding and genotyping of the mutant lines were performed according to previous publications: $CB1^{loxP/loxP;Nex\text{-}cre}$ mice (referred to as Glu-$CB1^{-/-}$ mice; [10]), $CB1^{loxP/loxP;Dlx5/6\text{-}cre}$ mice (referred to as GABA-$CB1^{-/-}$ mice; [10,21]), and $CB1^{loxP/loxP;D1\text{-}cre}$ mice (referred to as D1-$CB1^{-/-}$ mice; [11]). While Glu-$CB1^{-/-}$ mice lack the CB1 receptor in cortical glutamatergic neurons, GABA-$CB1^{-/-}$ mice lack the CB1 receptor specifically in GABAergic neurons [10]. In D1-$CB1^{-/-}$ mice, the CB1 receptor inactivation can primarily be found in GABAergic striatal medium spiny neurons, but also in a minor fraction of glutamatergic neurons in layer VI of the neocortex [11]. Wild-type littermates do not possess the respective Cre recombinase transgenic allele, and contain the CB1 floxed allele in a homozygous state. These mice were referred to as Glu-$CB1^{+/+}$, D1-$CB1^{+/+}$ and GABA-$CB1^{+/+}$. All mutant lines were bred for >10 generations on the background of C57BL/6N mice from Charles River, Germany. For detailed information on the anatomical differences in CB1 receptor expression, see Monory et al. [11].

Experimental design

Animals were group-housed (3–5 animals per cage type 2 (26.5×20.5×14.0 cm), EBECO Germany) until one week before behavioural testing. Animals were then separated and single-housed to avoid behavioural differences between dominant and subordinate animals. The same animals were used in each paradigm. Between each experimental paradigm, animals were allowed to rest for one week. All experiments were performed one hour after turning off the lights (2 pm), in the active phase of the animals, with only a minimal red light source in the room (0 lux).

Open Field and Novel Object Recognition Task

The novel object recognition task combines a general exploration test with a visual recognition memory paradigm. Therefore, it is used to evaluate object exploration and object recognition. The test was performed in a white plastic open field chamber (H40 cm×W40 cm×L40 cm). The protocol used was modified from Ennaceur and Delacour , Tang et al., and Tordera et al. [22–24].

For habituation, the animals were placed into the empty open field and allowed to explore the box for 10 min once a day for two days. The first habituation session was analyzed according to a standard open field paradigm, hence, total distance moved and time spent in the center (defined as 20 cm×20 cm) was evaluated using SMART software (PanLab, Spain). On day 3, two identical objects (O1 left, and O1 right; two metal cubes with H4 cm×W3 cm×L5 cm) were placed symmetrically 6–7 cm from the walls and separated 16–18 cm from each other. The mouse was placed into the box at an equal distance from both objects and video-recorded for 10 min. After this first exposure to the object, the mouse was returned to its home cage. 2 h and 24 h later, the mouse was placed again into the open field and exposed to the familiar object (O1) and to a novel object (O2 for the 2 h time point, and O3 for the 24 h time point, respectively) each time for 10 min (retention tests). The novel object O2 was a plastic billiard ball (5.72 cm in diameter) fixed on a metal plate (0.2 cm) and O3 was a round glass flask (H6 cm×W3 cm), filled with sand and closed with a black rubber plug. The familiar object was always positioned on the left side, while the new object was on the right side. Box and objects were cleaned with 70% ethanol after each trial to avoid olfactory cues. Experiment was video-recorded and the total time that the animal spent exploring each of the two objects in training and retention phase was evaluated by an experimenter blind to the genotype. Object exploration was defined as the orientation of the nose directly to the object at a distance <2 cm and/or touching the object with the nose and whiskers. Time spent climbing and sitting on the object were not regarded as exploration, and was therefore excluded from measurement [22], as these activities do not present a form of exploration. The discrimination index (DI) was calculated as the difference between the time spent exploring the new (N) and familiar (F) object, divided by the total time exploring the objects [(N−F)/(N+F)]. A positive DI is considered to reflect increased memory retention for the familiar object [24].

Sociability Test

A modified sociability test was performed, based on a published protocol [25]. In short, the test chamber (H41 cm×W42 cm×L70 cm) was divided into three compartments (H40 cm×W40 cm ×L22 cm), all accessible by openings (H7.5 cm×W10 cm) in the

dividing walls. Chambers and cages were cleaned with 70% ethanol between each trial to avoid olfactory cues. Experiment was video-recorded, and the total time that the test animals spent in each of the compartments during sociability and social novelty phase was measured by SMART software (PanLab, Spain). Male C57BL/6N animals (10–12 weeks old) were used as interaction partners for the sociability and social novelty phase.

Habituation Phase. The test animal was placed into the middle compartment for 5 min with entries to the side compartments blocked.

Sociability Phase. After the habituation phase, blockades of the entries were removed, allowing free access to the side compartments for 10 min. By doing this, the animal tested was exposed to a novel C57BL/6N interaction partner and a novel object (round cage described below), positioned in the two side compartments. The position of the interaction partner (left vs. right compartment) was alternated between trials to avoid any bias. The interaction partner itself was enclosed in a round cage (10 cm in diameter; 30 cm high [upper 20 cm Plexiglass, lower 10 cm covered by metal bars 1 cm apart to allow interaction but prevents fighting]). To minimize stress levels of the animals used as interaction partners, they were habituated to the cages four times for 10 min distributed over two days prior to the actual test days. To counterbalance individual differences of these interaction partners they were equally used for wild-type and mutant test mice. The novel object control (empty cage, no animal) was always positioned in the opposite compartment to the cage with the interaction partner. The discrimination index (DI) was calculated as the difference between the time spent exploring the novel object (nO) and the novel animal (nA), divided by the total time exploring both [(nO−nA)/(nO+nA)]. A positive DI is considered to reflect increased preference for the social interaction partner.

Social Novelty Phase. 2 h after the sociability phase, an additional, unknown interaction partner (novel) was introduced. The interaction partner from the sociability phase (familiar) was again placed into the same cage and same compartment as before. The novel animal was placed into the former empty cage and positioned at the respective side compartment. Openings were unblocked. The test animal was placed into the middle compartment, and the test animal was allowed to freely explore for 10 min. The DI was calculated as the difference between the time spent exploring the new (N) and the familiar (F) animal, divided by the total time exploring both [(N−F)/(N+F)]. A positive DI is considered to reflect increased memory retention for the familiar animal.

Resident-Intruder Test

The resident-intruder test was performed by placing a novel, group-housed intruder into the home cage of the test animal for 10 min. This paradigm allows evaluating social exploration and aggressive behaviour [26]. To decrease interaction induced by the intruder, younger animals (males, 11–13 weeks) were used as intruders. Experiment was video-recorded, and the total interaction time of the animals spent exploring was measured by an experimenter blind to the genotype. Interaction was defined by any type of physical interaction induced by the resident clearly directed towards the partner. Duration, percentage of time and number of fights were evaluated separately. Fighting was defined by physical struggling between the interaction partners initiated by an attack of the resident towards the intruder.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of individual data points. Results were considered to be significant at $p<0.05$. All behavioural endpoints of the novel object recognition task were initially analyzed using two-way ANOVA, using genotype and object as variables and Bonferroni post-tests to correct for multiple comparisons. In some cases, to analyze the locomotion effects in the open field, the sociability in the sociability test and the aggression in the resident-intruder paradigm for each genotype, data were analysed using an unpaired Student's t-test or Kruskal-Wallis statistic. Additionally, in order to evaluate whether the DI of the genotypes deviated significantly from zero, we used the unpaired t-test with Welch's correction. Graphs and statistics were generated by GraphPad Prism 4.03 (GraphPad Software; http://www.graphpad.com).

Results

Open Field

The evaluation of the locomotor activity in the open field revealed that only the GABA-CB1$^{-/-}$ mice showed an alteration ($T_{(18)} = 3.213$, $p = 0.0048$; Table 1). None of the other mutants showed any change in the distance moved compared with their respective wild-type littermates in the open field (Glu-CB1 line [$T_{(34)} = 1.609$, $p = 0.1169$]; D1-CB1 line [$T_{(21)} = 0.5618$, $p = 0.5802$]). In regard to the time spent in the center region of the open field, we could not detect an alteration in any of the mutants (Glu-CB1 line [$T_{(34)} = 0.8168$, $p = 0.4197$]; GABA-CB1 line [$T_{(18)} = 1.418$, $p = 0.1733$]; D1-CB1 line [$T_{(21)} = 0.9048$, $p = 0.3758$]; see Table 1).

Novel Object Recognition Task

The analysis of the novel object recognition task (referred to as NORT in Table 1) revealed a decrease in general object exploration in Glu-CB1$^{-/-}$ mice as compared to wild-type littermate controls (Fig. 1A,D,G). We detected a significant decrease in time spent with the objects O1 in the training session ($F_{(1,62)} = 4.183$, $p = 0.0451$; Fig. 1A), but also in the 2 h ($F_{(1,66)} = 13.68$, $p = 0.0004$; Fig. 1D) and 24 h retention sessions ($F_{(1,66)} = 32.87$, $p<0.0001$; Fig. 1G) for the novel object O2. In contrast, GABA-CB1$^{-/-}$ mice displayed a general increase in exploration in all the sessions as compared to controls (training [$F_{(1,74)} = 17.88$, $p<0.0001$], 2 h retention [$F_{(1,74)} = 8.411$, $p = 0.0049$)], 24 h retention [$F_{(1,74)} = 6.172$, $p = 0.0152$]; Fig. 1B,E,H). In the D1-CB1 mutant line, no genotype differences were observed in the general object exploration (training [$F_{(1,44)} = 1.760$, $p = 0.1915$], 2 h retention [$F_{(1,44)} = 0.08051$, $p = 0.7721$], 24 h retention [$F_{(1,44)} = 3.317$, $p = 0.0754$]; Fig. 1C,F,I).

Evaluation of the discrimination index (DI) revealed that all groups, independent of the line, showed no differences within the training session regarding the exploration of the left and the right object O1, respectively. (Glu-CB1$^{+/+}$ [$T_{(20)} = 0.8230$, $p = 0.4202$]; Glu-CB1$^{-/-}$ [$T_{(11)} = 0.9582$, $p = 0.3585$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 1.118$, $p = 0.2812$]; GABA-CB1$^{-/-}$ [$T_{(22)} = 1.959$, $p = 0.0630$]; D1-CB1$^{+/+}$ [$T_{(11)} = 1.447$, $p = 0.1758$]; D1-CB1$^{-/-}$ [$T_{(11)} = 1.679$, $p = 0.1213$]; Table 1). Furthermore, no discrimination differences compared to their respective wild-type controls were found for all mutants within the training session (Glu-CB1 line [$T_{(31)} = 1.407$, $p = 0.1693$]; GABA-CB1 line [$T_{(37)} = 0.06488$, $p = 0.9486$]; D1-CB1 line [$T_{(22)} = 1.951$, $p = 0.0639$]; Table 1).

In the 2 h retention phase, several groups lacked a significant discrimination between the familiar and the novel object. Only Glu-CB1$^{+/+}$, D1-CB1$^{+/+}$ and D1-CB1$^{-/-}$ animals displayed a significant preference towards the novel stimulus (Glu-CB1$^{+/+}$ [$T_{(21)} = 4.806$, $p<0.0001$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 1.220$, $p = 0.2458$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 0.07097$, $p = 0.9444$]; GABA-CB1$^{-/-}$ [$T_{(22)} = 1.366$, $p = 0.1858$]; D1-CB1$^{+/+}$ [$T_{(10)} = 2.502$, $p = 0.0313$]; D1-CB1$^{-/-}$ [$T_{(10)} = 2.238$, $p = 0.0492$]; Table 1). Comparison

Table 1. Locomotion, anxiety and memory.

	Glu-CB1		GABA-CB1		D1-CB1	
Paradigm	**+/+**	**−/−**	**+/+**	**−/−**	**+/+**	**−/−**
	Distance Moved (cm)					
Open Field	2824±209	2324±182	2621±306	3801±202**	4456±91	4368±131
Sociability						
Habituation	2012±113	1569±162*	1597±59	1730±64	1669±81	1850±118
Sociability	5171±205	4891±312	4945±127	5083±175	5055±162	5079±157
Social Novelty	3973±211	3125±227*	4023±141	4623±175*	3863±292	3942±160
	Time in Center (sec)					
Open Field	63.3 ± 12	80.9±20	156.0±48	82.0±21	65.4±11	82.4±15
	Discrimination Index (DI)					
NORT						
Training	0.01±0.01	−0.08±0.08	−0.03±0.03	−0.03±0.02	−0.08±0.05	0.03±0.02
Retention 2 h	0.16±0.03#	0.06±0.05	0.00±0.06	−0.03±0.03	0.15±0.06#	0.08±0.03#
Retention 24 h	0.25±0.06#	0.06±0.13	0.18±0.06#	0.15±0.05#	0.27±0.08#	0.18±0.05#
Sociability						
Sociability	0.29±0.03#	0.12±0.07*	0.27±0.03#	0.35±0.04#	0.20±0.04#	0.30±0.04#
Social Novelty	0.05±0.03	0.08±0.09	−0.01±0.05	0.09±0.03#	0.03±0.06	0.03±0.06

Evaluation of locomotion (distance moved), anxiety (time in center) and memory (discrimination index) for all mutant lines; $^{+/+}$ (wild-type), $^{-/-}$ (mutant); t-test analysis:
*$p<0.05$;
**$p<0.01$ (significance between genotype);
#$p<0.05$ (significant from 0; positive recognition of novel object).
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between the mutants and their respective wild-type littermates displayed no significant differences in all lines (Glu-CB1 line [$T_{(33)} = 1.775$, $p = 0.0850$]; GABA-CB1 line [$T_{(37)} = 0.6235$, $p = 0.5368$]; D1-CB1 line [$T_{(20)} = 0.9965$, $p = 0.3309$]; Table 1).

In the 24 h retention phase, independently of the genotype, all groups showed a significant preference towards the novel object, with the only exception of the Glu-CB1$^{-/-}$ animals (Glu-CB1$^{+/+}$ [$T_{(21)} = 4.472$, $p = 0.0002$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 0.4328$, $p = 0.6729$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 2.818$, $p = 0.0129$]; GABA-CB1$^{-/-}$ [$T_{(22)} = 3.072$, $p = 0.0056$]; D1-CB1$^{+/+}$ [$T_{(11)} = 3.601$, $p = 0.0042$]; D1-CB1$^{-/-}$ [$T_{(11)} = 3.540$, $p = 0.0046$]; Table 1). Comparison between the mutants and their respective wild-type littermates diplayed no genotype difference (Glu-CB1 line [$T_{(33)} = 1.522$, $p = 0.1374$]; GABA-CB1 line [$T_{(37)} = 0.1255$, $p = 0.9008$]; D1-CB1 line [$T_{(22)} = 1.049$, $p = 0.3055$]; Table 1).

The evaluation of object specific exploration (O1 left or O1-3 right) over the three sessions (training, 2 h retention and 24 h retention), revealed a significant difference for the Glu-CB1$^{-/-}$ as compared to their littermate controls. Thus, the Glu-CB1$^{-/-}$ mutants showed a steadily decreasing investigatory behaviour for both, the left object (increasing familiarity) and the right object (always novel) (Glu-CB1$^{-/-}$ interaction [object/time]: $F_{(2,48)} = 0.1537$, $p = 0.8580$; Bonferroni post-test: training $p>0.05$, 2 h $p>0.05$, 24 h $p>0.05$; Fig. 1A,D,G). This phenomenon was only seen in the Glu-CB1$^{+/+}$ mice for the left object (increasing familiarity), while the time spent investigating the right object (always novel) remained constant (Glu-CB1$^{+/+}$ interaction [object/time]: $F_{(2,84)} = 4.851$, $p = 0.0101$; Bonferroni post-test: training $p>0.05$, 2 h $p>0.05$, 24 h $p<0.01$; Fig. 1A,D,G). It was further possible to detect a significant difference between the genotypes in exploring the right object, but not the left object over the three sessions (left object interaction [genotype/time]: $F_{(2,48)} = 0.2283$, $p = 0.7965$; Bonferroni post-test: training $p>0.05$, 2 h $p>0.05$, 24 h $p>0.05$; right object interaction [genotype/time]: $F_{(2,66)} = 3.522$, $p = 0.0352$; Bonferroni post-test: training $p>0.05$, 2 h $p<0.05$, 24 h $p<0.001$; Fig. 1A,D,G).

Sociability Test

During the sociability phase, the Glu-CB1$^{-/-}$ animals showed a significant increase in time spent in the middle compartment ($T_{(33)} = 2.247$, $p = 0.0314$; Fig. 2A). Accordingly, these mutants displayed a significant decrease in time spent with the interaction partner but not with the object (mouse [$T_{(33)} = 3.734$, $p = 0.0007$]; object [$T_{(33)} = 1.412$, $p = 0.1672$]; Fig. 2A). A similar result was obtained, when the novel interaction partner was introduced during the social novelty test. While the Glu-CB1$^{-/-}$ mice spent more time in the middle compartment, they spent less time with the familiar and novel partner as compared to the wild-type littermates (middle [$T_{(33)} = 3.772$, $p = 0.006$]; familiar [$T_{(33)} = 2.263$, $p = 0.0303$]; unknown [$T_{(33)} = 2.596$, $p = 0.0140$]; Fig. 2D). This phenotype was opposite to the findings with the GABA-CB1 line. In the sociability phase as well as in the social novelty phase, the GABA-CB1$^{-/-}$ mice showed a significant increase in time spent with the novel interaction partner as compared to controls (sociability [$T_{(57)} = 2.099$, $p = 0.0403$]; social novelty [$T_{(35)} = 3.063$, $p = 0.0042$]; Fig. 2B,E). The time spent in the middle compartment was consequently decreased (sociability [$T_{(57)} = 2.740$, $p = 0.0082$]; social novelty [$T_{(35)} = 2.168$, $p = 0.037$]). Interestingly, the time spent in the compartment with the empty cage (i.e. the object only) during the sociability phase as well as the time spent with the familiar animal (social novelty test) were not different between mutants and controls (object [$T_{(57)} = 1.114$, $p = 0.2699$]; familiar [$T_{(35)} = 1.017$, $p = 0.3162$]; Fig. 2B,E). The analysis of the D1-CB1 line did not reveal any significant genotype differences in the 3 phases of the sociability test (Fig. 2C,F). Only a non-significant trend was observed in the

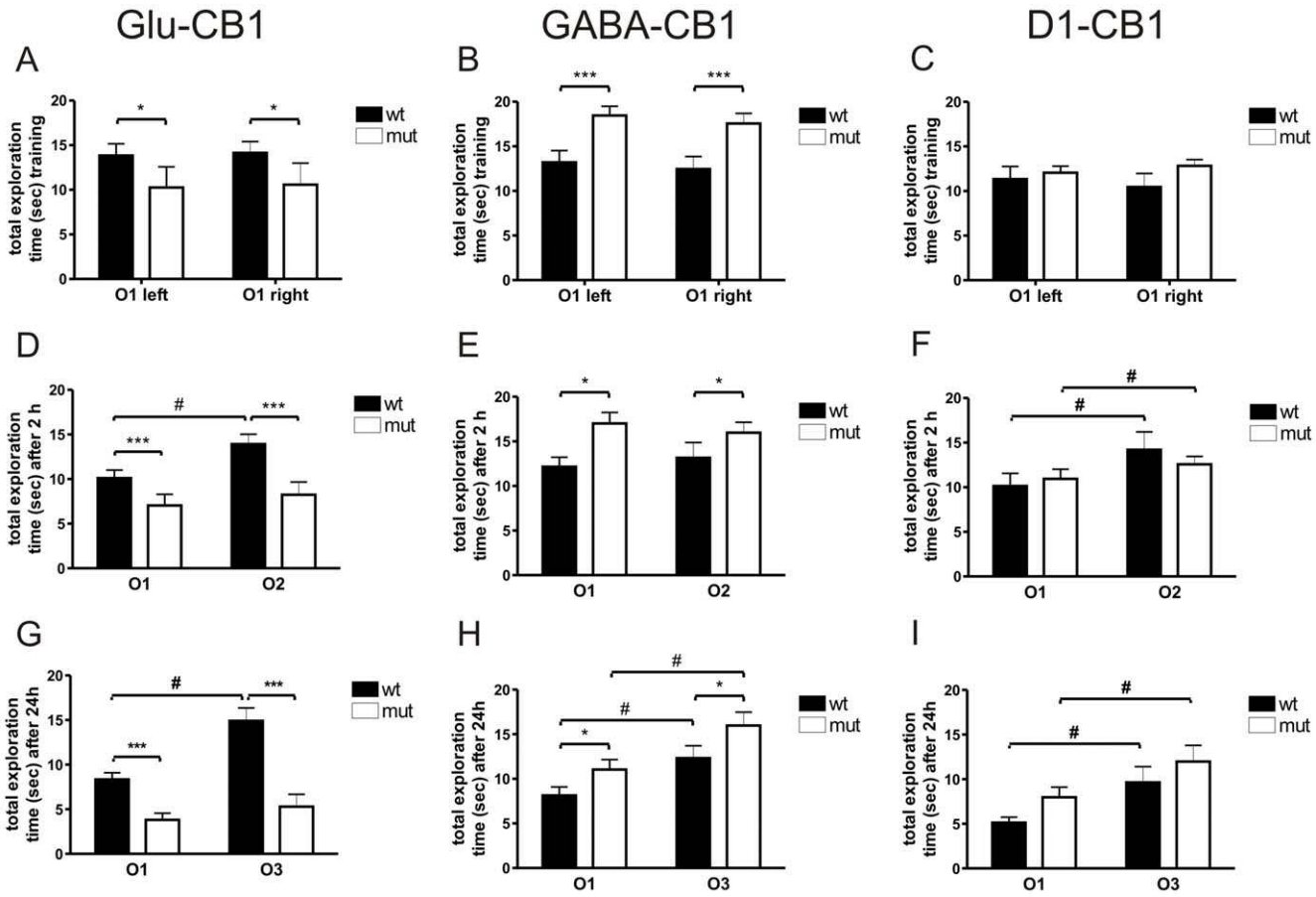

Figure 1. Inanimate exploration in the novel object recognition task. (A–C) Total time of exploration of two identical objects (O1, both on left and right side) during the training session for three conditional CB1 receptor mutant lines (Glu-CB1 [n = 23+13], GABA-CB1 [n = 18+23], D1-CB1 [12+12]) and their wild-type control littermates. (D–F) Total time of exploration of familiar object (O1) and novel object (O2 or O3) during the retention session after 2 h or 24 h (G–I). Glu-CB1$^{-/-}$ mice displayed a reduced exploration, while GABA-CB1$^{-/-}$ mice showed an increased exploration both in the training and retention session when compared to their wild-type littermate controls. No significant genotype differences were observed in the D1-CB1 mutant line. 2-way ANOVA (genotype differences) *p<0.05, ***p<0.001; t-test (discrimination index DI) $^{\#}$p<0.05.
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sociability phase. Even though time spent in the middle area was not altered ($T_{(28)} = 0.9190$, $p = 0.3659$), it seemed that the mutants showed a slight preference to explore the interaction partner ($T_{(28)} = 1.909$, $p = 0.0666$) rather than the empty cage ($T_{(28)} = 1.859$, $p = 0.0736$), i.e. the object (Fig. 2C). In the social novelty phase, not even a trend was detectable (familiar [$T_{(30)} = 0.7636$ $p = 0.4511$]; novel [$T_{(30)} = 0.5840$ $p = 0.5636$]; middle [$T_{(30)} = 0.6112$ $p = 0.5457$]; Fig. 2F).

The evaluation of the DI showed only minimal differences between the genotypes. In the sociability phase, the Glu-CB1$^{-/-}$ animals showed an impaired preference towards the interaction partner as compared to their controls ($T_{(33)} = 2.537$, $p<0.0161$; Table 1). In contrast, the GABA-CB1$^{-/-}$ mice and the D1-CB1$^{-/-}$ mice showed no significant changes in the preference towards the interaction partner (GABA-CB1$^{-/-}$ [$T_{(57)} = 1.507$, $p<0.1373$], D1-CB1$^{-/-}$ [$T_{(28)} = 1.636$, $p<0.1130$]; Table 1). In the social novelty phase, no DI differences were observed in any of the lines (Glu-CB1 line [$T_{(33)} = 0.3977$ $p = 0.6934$]; GABA-CB1 line [$T_{(34)} = 1.794$ $p = 0.0817$]; D1-CB1 line [$T_{(30)} = 0.6126$ $p = 0.547$]).

For all lines and genotypes, except for the Glu-CB1$^{-/-}$ mice, we observed a strong preference towards the social interaction partner over the object in the sociability phase (Glu-CB1$^{+/+}$ [$T_{(21)} = 10.47$, $p<0.0001$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 1.559$, $p = 0.1450$]; GABA-CB1$^{+/+}$ [$T_{(27)} = 8.309$, $p<0.0001$]; GABA-CB1$^{-/-}$ [$T_{(30)} = 8.187$, $p<0.0001$]; D1-CB1$^{+/+}$ [$T_{(16)} = 5.017$, $p = 0.0002$]; D1-CB1$^{-/-}$ [$T_{(13)} = 7.458$, $p<0.0001$]; Table 1). In the social novelty phase, none of the groups, except for the GABA-CB1$^{-/-}$ mice, showed any preference towards the novel over the familiar interaction partner (Glu-CB1$^{+/+}$ [$T_{(21)} = 1.453$, $p<0.1610$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 0.8652$, $p = 0.4039$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 0.2402$, $p = 0.8134$]; GABA-CB1$^{-/-}$ [$T_{(19)} = 2.674$, $p = 0.0150$]; D1-CB1$^{+/+}$ [$T_{(16)} = 0.4262$, $p = 0.6756$]; D1-CB1$^{-/-}$ [$T_{(14)} = 0.4437$, $p = 0.6841$]; see Fig. 1).

The evaluation of the locomotor activity revealed no significant changes in the habituation phase of the sociability test, except for the Glu-CB1$^{-/-}$ mice, which showed a decrease in locomotion (Glu-CB1 line [$T_{(33)} = 2.312$, $p = 0.0271$]; GABA-CB1 line [$T_{(60)} = 1.506$, $p = 0.1374$]; D1-CB1 line [$T_{(29)} = 1.571$, $p = 0.1270$]). In the sociability phase, no alteration in the distance moved was observed in any of the lines (Glu-CB1 line [$T_{(29)} = 0.7833$, $p = 0.4398$]; GABA-CB1 line [$T_{(62)} = 0.6159$, $p = 0.5402$]; D1-CB1 line [$T_{(30)} = 0.1082$, $p = 0.9145$]). However, a significant decrease and increase in the distance moved was detected in the social novelty phase for the Glu-CB1$^{-/-}$ mice and the GABA-CB1$^{-/-}$ mice, respectively (Glu-CB1 line [$T_{(33)} = 2.575$, $p = 0.0146$]; GABA-CB1

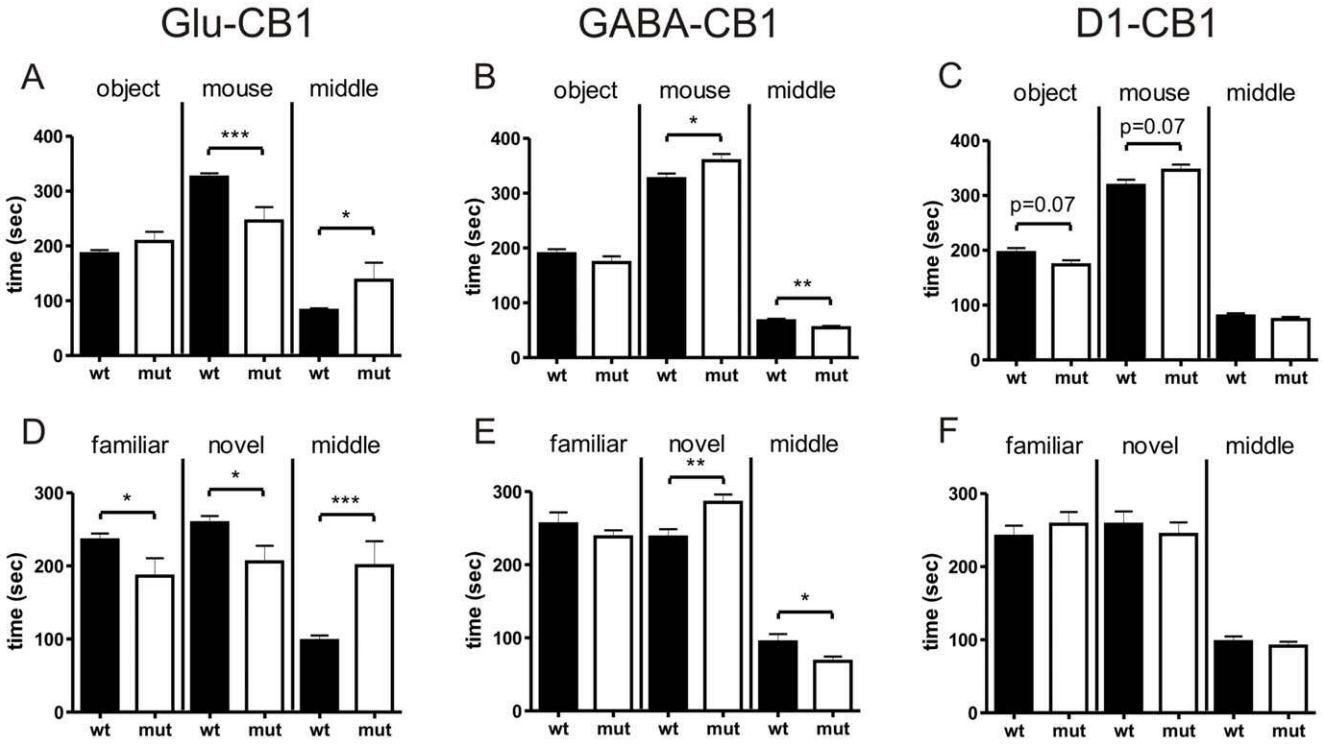

Figure 2. Animate vs. inanimate exploration in the sociability test. (A–C) Comparison of animate (mouse) and inanimate (object, ''empty'') exploration for the three mutants lines (Glu-CB1 [n = 22+13], GABA-CB1 [n = 18+23], D1-CB1 [16+16]) and their wild-type littermate controls during the sociability phase. (D–F) Exploration of the familiar and the novel interaction partner for during the social novelty phase. Glu-CB1$^{-/-}$ mice displayed no significant change in the exploration session, where there was a choice between the object and the interaction partner. In the social novelty phase, however, the interaction with a novel interaction partner was decreased when compared with their wild-type littermate controls. GABA-CB1$^{-/-}$ mice showed an increased social interaction in both sessions. In the D1-CB1 mutant line, no genotype differences were observed neither in the sociability nor in the social novelty phase. n = 11–20 animals; t-test *p<0.05, **p<0.01.
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line [$T_{(38)} = 2.591$, $p = 0.0135$]). The D1-CB1$^{-/-}$ mice again showed no change in the distance moved as compared to their respective wild-type littermates ($T_{(30)} = 0.2386$, $p = 0.8130$).

Resident-Intruder Test

Glu-CB1$^{-/-}$ mice displayed a significant decrease interacting with the intruder animals for the 10 min interaction phase as compared with wild-types ($T_{(35)} = 2.297$, $p = 0.0277$). Splitting the 10 min period into two 5 min bins revealed that the difference in interaction was mainly visible for the first 5 min bin ($T_{(35)} = 3.106$, $p = 0.0038$) (Fig. 3A). In addition, Glu-CB1$^{-/-}$ mice displayed an altered aggressive behaviour. Even though the number of fights was not different between the genotypes, the time that Glu-CB1$^{-/-}$ mice spent fighting the intruder was increased ($T_{(35)} = 2.249$, $p = 0.0309$) (Fig. 3D). As observed in the previous experiments, we detected an opposite phenotype in the GABA-CB1$^{-/-}$ animals, which showed an increased interaction with the intruder animal ($T_{(29)} = 2.522$, $p = 0.0174$) (Fig. 3B). The overall fighting with the younger intruder did not change as compared to the wild-type littermates ($T_{(26)} = 0.4227$, $p = 0.6760$, $T_{(29)} = 0.6286$, $p = 0.5345$) (Fig. 3E). D1-CB1$^{-/-}$ mice again displayed no phenotype differences, neither in interaction time spent with the intruder ($T_{(20)} = 0.3481$, $p = 0.7314$), nor in fighting behaviour ($T_{(22)} = 0.0000$, $p = 1.0$, $T_{(22)} = 0.8261$, $p = 0.4176$) (Fig. 3C,F).

Additional analysis revealed that Glu-CB1$^{+/+}$ animals displayed a significant increase in aggression as compared to the other control groups, GABA-CB1$^{+/+}$ and D1-CB1$^{+/+}$. Thus, differences were detected in number of fights (Kruskal-Wallis statistic = 7.478, $p = 0.0238$; Dunn's Multiple Comparison Post-Test: Glu-CB1$^{+/+}$ vs GABA-CB1$^{+/+}$ $p<0.05$, Glu-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$, GABA-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$), as well as % of time fighting (Kruskal-Wallis statistic = 7.584, $p = 0.0226$; Dunn's Multiple Comparison Post-Test: Glu-CB1$^{+/+}$ vs GABA-CB1$^{+/+}$ $p<0.05$, Glu-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$, GABA-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$).

Discussion

Using different conditional CB1 receptor mutant mice, we were able to show that the deletion of the CB1 receptor from forebrain GABAergic or cortical glutamatergic neurons resulted in an opposite behavioural outcome regarding animate and inanimate exploration. On the other hand, deletion of the CB1 receptor from dopamine receptor D1-expressing GABAergic striatal medium spiny neurons did not result in any significant changes. These findings suggest a regulatory function of the eCB system in cortical GABAergic and glutamatergic circuits to prevent neuronal and behavioural imbalance.

Mice lacking the CB1 receptor on glutamatergic neurons displayed a decreased exploratory behaviour, both in animate interaction (the interaction with a partner) and inanimate interaction (the interaction with an object). A similar decrease in object and social exploration was found in earlier studies, which were related with increased fear [6,7]. In our study, the decrease in exploration was seen when the mouse was exposed to a social interaction partner and/or to an object, and seemed to be

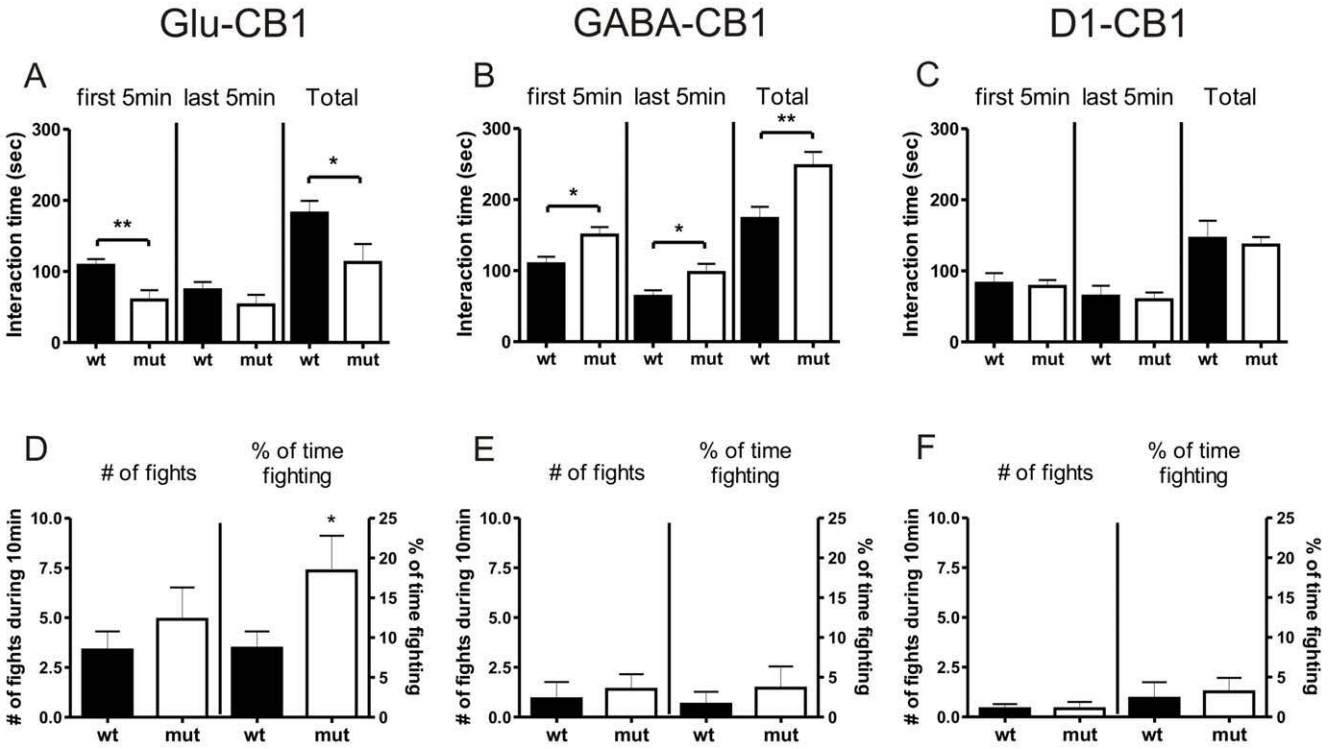

Figure 3. Animate exploration in the resident-intruder test. (A–C) Social interaction with an unknown, younger intruder for all three mutant lines (Glu-CB1 [n = 23+13], GABA-CB1 [n = 18+23], D1-CB1 [n = 16+16]). (D–E) Number of fights induced by the resident is shown for all three mutant lines. Glu-CB1$^{-/-}$ mice showed a significantly reduced exploration during the first 5 min observation period and an increased aggression towards the intruder when compared to wild-type littermate controls. GABA-CB1$^{-/-}$ mice displayed an increased interaction with the intruder, but no difference in aggressive behaviour. D1-CB1$^{-/-}$ mice showed no behavioural changes as compared to their wild-type littermate controls. t-test *$p<0.05$, **$p<0.01$.
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independent of novelty (Figs. 1, 2D). This phenotype was also visible in the resident-intruder test. However, the decreased social investigation was mainly based on a lower exploration during the first 5 min interval, a period important for information gathering (Fig. 3A).

The anxiogenic-like behaviour associated to these mutants can also explain the significantly higher aggression level found in the resident-intruder paradigm (Fig. 3D), a behaviour which was also observed in complete CB1 receptor knock-out animals [27]. The age and strength of the intruder compared to the resident is highly important [28]. In our case, the intruders were weaker and should not be regarded as a threat. We would therefore suggest that the deletion of the CB1 receptor from glutamatergic neurons might result in an inadequate aggressive response, suggesting an important role of CB1 receptor on this neuronal population in aggression. CB1 receptor in cortical GABAergic interneurons appears to mediate an opposite behaviour. While D1-CB1$^{-/-}$ animals (CB1 receptor loss primarily in the striatum), did not reveal any significant difference as compared to wild-type littermates, we observed that GABA-CB1$^{-/-}$ mice (lacking CB1 receptor additionally from cortical GABAergic interneurons), showed an increase in animate and inanimate exploration. Accordingly, increased investigatory behaviour toward novel food or object was previously observed in the GABA-CB1$^{-/-}$ mice [6]. Interestingly, Glu-CB1$^{+/+}$ control animals displayed an increased aggressive behaviour in the resident intruder paradigm as compared to the other wild-type controls, GABA-CB1$^{+/+}$ and D1-CB1$^{+/+}$. This elevated aggression might be explained by the fact that both the wild-type and mutant littermates are group-housed during growth. In case of the Glu-CB1 line, the modulated social behaviour of the Glu-CB1$^{-/-}$ mutants might have an effect on their wild-type littermates.

Taken together, these results suggest an anxiolytic-like function of the CB1 receptor on glutamatergic neurons and an anxiogenic-like function of the CB1 receptor on GABAergic interneurons. However, a generalized conclusion on the involvement CB1 receptor on cortical glutamatergic neurons in anxiety is not yet possible to be drawn, as under our experimental conditions, the open field test was not congruent with this notion. Neither Glu-CB1$^{-/-}$ nor GABA-CB1$^{-/-}$ mutants spend a different period of time in the more aversive center zone as compared with their respective wild-type littermates (Table 1). In addition, studies with these animals on the elevated plus maze, an anxiety test, did not reveal any changes either [7,14]. Also, levels of corticosterone under basal and stressful conditions were found to be similar between mutant and wild-type controls in both mutant lines [29]. Thus, it seems that a respective exploratory stimulus, such as an object or interaction partner, is required to induce a phenotype in these mice.

An alternative explanation for the observed differences can be alterations in spontaneous locomotor activity. In fact, we observed for both the Glu-CB1$^{-/-}$ and GABA-CB1$^{-/-}$ changes in the distance moved, namely a decrease and increase, respectively. It seems unlikely that the difference in locomotion was the driving force underlying the exploration phenotypes, as the mutants, in contrast to the variation in animate and social investigation, did not always display the locomotor alterations (Table 1). We argue that a respective context (e.g. handling threshold, exploratory

stimulus) is required for a detectable locomotion phenotype in our mutant lines. A similar situation seems to be true for the general investigatory drive. Thus, the clear differences in exploring object or interaction partner is not mirrored by the findings in the open field test, where we were not able to detect any alteration in the time spent in the more aversive center region (Table 1). This notion is supported by other studies with these mutant lines, where a behavioural change is only detectable in the presence of a respective stimulus or pharmacological modification of the eCB system [6,7].

A further explanation for the behavioural differences might be memory alterations in the respective mutant. However, this might only account for the Glu-CB1$^{-/-}$ mutants, as all other animals, independently of line and genotype, displayed a similar memory and recognition performance. Especially after 24 hours, mice recognized and distinguished strongly between familiar and novel objects (Table 1). The low discrimination index to the familiar object after a 2 hour interval in several groups, however, is unexpected and cannot be explained at this point. Only Glu-CB1$^{-/-}$ failed to show a clear preference towards the novel object in both retention sessions, indicating a memory deficit. Problematic for the interpretation is the overall low exploration for this mouse line, which is true for all three sessions of the novel object recognition test, as well as the other behavioural paradigms. Of special interest is the altered behaviour of the mutants in response to the novel objects. While wild-type littermates displayed a constant interest for the novel objects (O1–O3), the Glu-CB1$^{-/-}$ animals showed a steadily decreasing exploration over the three sessions (Fig. 1A,D,G). For both genotypes, such a decrease was seen regarding the exploration of the familiar objects (O1), which is not surprising, as novelty of this object strongly decreased with each session. Thus, the Glu-CB1$^{-/-}$ mice appeared to respond to the familiar and novel object in a similar way, suggesting rather a habituation to the context than a memory deficit. Nevertheless, a final conclusion cannot be made.

As mentioned above, all groups, independently of the line and the genotype, showed a stronger preference for the social interaction partner as compared to the object in the sociability test (Table 1). This behaviour was expected, as animals normally prefer social over non-social contacts [25]. Surprisingly, we could not detect a significant preference towards the novel interaction partner in the social novelty phase (Table 1). While this preference was observed in several lines [25], in our hands it was only recognizable in the GABA-CB1$^{-/-}$ mice. This finding could indicate that social discrimination is impaired in these mutants. However, comparable results from other studies suggest that a strong social preference does not necessarily predict a strong preference for social novelty. As a matter of fact, two different components of social behaviour were postulated to underlie sociability and social novelty, respectively. In addition, life history and development are responsible for lower or higher novelty preference [30].

Taken together the strong differences observed in the GABA-CB1$^{-/-}$ and Glu-CB1$^{-/-}$ animals in respect to their wild-type littermates might be explained by anxiolytic and anxiogenic responses to novelty, respectively. Nevertheless, the eCB system has also been shown to be involved in learning and memory function, which should be kept in mind here [31,32]. It may be even likely that both anxiety and memory components function together in our paradigms, but to solve this issue would require further investigations using other behavioural paradigms.

Our results, namely the increase of exploration following the deletion of GABAergic CB1 receptor and the decrease of exploratory behaviour following the deletion of glutamatergic CB1 receptors, may explain the contradictory findings using Δ^9-THC, URB597 and VDM11, as described in above. We suggest that increased or decreased exploratory drive, respectively, as response to cannabinoid treatment depends on the predominant modulation of either GABAergic or glutamatergic CB1 receptor, e.g. the activation of GABAergic CB1 receptor decreases exploration, while the activation of glutamatergic CB1 receptor leads to an increased investigatory drive. Thus, the decreased exploration induced by chronic and systemic activation of the eCB system with Δ^9-THC might be due to the exogenous activation of the CB1 receptor in GABAergic interneurons [15–17]. The increased exploratory profile after inhibition of anandamide degradation or reuptake could be explained by a specific on-demand activation of the CB1 receptor on glutamatergic neurons [17]. On the other hand, the increased animate and inanimate interaction as a result of the complete deletion of the CB1 receptor might be caused by the increased GABAergic drive [7,20]. It seems that the GABAergic drive is the predominant factor for behavioural outcome, when the eCB system is activated or blocked in a chronic manner. This makes the increased social interaction after URB597 treatment even more interesting, as in this case, the glutamatergic drive seems to be the predominant component. To test this hypothesis, Glu-CB1$^{-/-}$ or GABA-CB1$^{-/-}$ have to be injected with the respective drugs in comparable doses and tested in behavioural paradigms. Similar contradictory results were observed in pharmacological studies on anxiety and stress levels after cannabinoid administration, both being strongly involved in investigatory and exploratory drive [33,34]. The opposite effects might also be based on cortical GABAergic or glutamatergic transmission. Therefore, depending on its specific spatiotemporal activation within neuronal circuits, this system can act as a major "bi-directional" neuromodulator [14,34].

Our results might also be interesting in respect to some disorders, which are associated with inappropriate exploratory drive. Thus, a direct and indirect relation between these disorders and a dysregulation of GABAergic and/or glutamatergic transmission can be proposed. In animal models for autism, modulation of GABAergic transmission seems to be important [35,36]. The induction of schizophrenia-like symptoms by administration of the NMDA receptor antagonist phencyclidine revealed an alteration of glutamatergic and GABAergic signalling in the prefrontal cortex [37]. Interestingly, the effects of phencyclidine could be blocked by CB1 receptor antagonist treatment [38]. It was further shown that down-regulation of cortical glutamatergic drive resulted in an increase in dopamine levels and a hyperactive phenotype, which could be blocked by cortical GABA receptor activation [39]. These findings indicate a cortical control in these neuronal disorders, caused also by imbalanced GABAergic and glutamatergic transmission, a mechanism also suggested by our findings. Recent publications even suggest glutamatergic, instead of dopaminergic transmission to be the major factor of schizophrenia [40].

In conclusion, our results indicate a major, but opposite role of the eCB system in cortical GABAergic and glutamatergic neurons in the regulation of exploration (Table 2). Hence, further investigations along this line should be able to detail the diverse effects of cannabinergic drugs on investigatory behaviour. As investigatory drive is often associated with impulsive behaviour, studies using respective paradigms would be of great interest. Lastly, in future studies, the regulatory properties of the eCB system on cortical excitatory and inhibitory drive should be exploited in psychiatric disorders, opening up a therapeutic avenue to restore a possible cortical imbalance pharmacologically.

Table 2. Summary of behavioural changes induced by conditional CB1 receptor deletion.

	Locomotion	Object Exploration	Social Exploration	Aggression
Wild-type	Normal	Normal	Normal	Normal
Glu-CB1$^{-/-}$	Decreased	Decreased	Decreased	Increased
GABA-CB1$^{-/-}$	Increased	Increased	Increased	Normal
D1-CB1$^{-/-}$	Normal	Normal	Normal	Normal

"Normal" refers to similar to the wild-type behaviour on spontaneous locomotor activity (locomotion), investigation of object (object exploration) or of interaction partner (social exploration) and fights initiated (aggression).
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Author Contributions

Conceived and designed the experiments: MH NK KM BL. Performed the experiments: MH NK. Analyzed the data: MH NK KM. Contributed reagents/materials/analysis tools: MH NK KM BL. Wrote the paper: MH NK KM BL.

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