FUNCTIONAL ANALYSIS OF THE ENDOCANNABINOID SYSTEM IN CONDITIONAL MOUSE MUTANTS

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Abbreviations

AA	Arachidonic acid
2-AG	2-Arachidonoyl glycerol
ACEA	Arachidonyl-2'-chloroethylamide
ACTH	Adrenocorticotropin hormone
AEA	Arachidonoyl ethanolamine (Anandamide)
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BCP	1-bromo-3-chloropropane
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
bp	Base pair
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine serum albumine
CamKII α	Calcium/calmodulin dependent protein kinase II α
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
cm	Centimeter
CNS	Central nervous system
CORT	Corticosterone
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor 1
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate gyrus
dH_2O	Deionized H_2O
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid

DTT	Dithiothreitol
EDTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FAAH	Fatty acid amid hydrolase
FAM	6-carboxyfluorescein
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
fl	Floxed (flanked by loxP sites)
g	Gravitational force
g	Gram
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GPR	G-protein coupled receptor
GR	Glucocorticoid receptor
h	Hour
HCl	Hydrochloric acid
HPA-axis	Hypothalamic–pituitary–adrenal axis
HRP	Horseradish peroxidase
i.p.	intraperitoneal
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
kHz	Kilohertz
LC	Liquid chromatography
LOX	Lipooxygenase
loxP	“locus of X-ing over” in Phage P1
LyzM	Lysozyme 2
M	Molar
MAGL	Monoacyl glycerol lipase
MDD	Major depression disorder

MeOH	Methanol
min	Minutes
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
ms	Milliseconds
MS	Mass spectrometry
n	Number (sample size)
NAc	Nucleus accumbens
NAPE	N-arachidonoyl-phoshatidylethanolamine
NAPE-PLD	NAPE-phospholipase D
NeuN	Neuronal nuclei
ng	Nanogram
nM	Nanomolar
ns	Not significant
NSC	Neural stem cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyd
PFC	Prefrontal cortex
PG	Prostaglandin
PI	Phosphatidylinositol
PI3K	Phosphatidylinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PVN	Periventricular nucleus
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
SAG	1-stearoyl-2-arachidonoyl-sn-glycerol
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean

SGZ	Subgranular zone
SSC	Saline sodium citrate
SVZ	Subventricular zone
Syn	Synapsin
TAG	Triacylglycerols
TE	Tris EDTA
THC	Δ9-tetrahydrocannabinol
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WT	Wild type
µl	Microliter
µM	Micromolar

Summary

The endocannabinoid system is an important modulatory and homeostatic system in the brain. Dysregulations of the endocannabinoid system are highly involved in affective disorders, such as anxiety-related disorders or major depression. For instance, pharmacological blockade or genetic deletion of the cannabinoid receptor 1 (CB1) increase the incidence and severity of affective disorders. In contrast to other neurotransmitters, endocannabinoids are produced on demand. To ensure proper endocannabinoid signaling, endocannabinoid levels in the brain need to be tightly controlled by synthesizing and metabolic enzymes. Recently, our institute has shown, that deletion of *Dagla*, the main producing enzyme of the endocannabinoid 2-AG, resulted in profound changes of anxiety-related and depression-like behaviors in mice. Yet, the cellular source of 2-AG in the brain is unknown. Moreover, it is not clear, which cell type is responsible for the phenotype observed in constitutive DAGLa knockout mice.

Thus, in the scope of this thesis conditional knockout mice with a cell-specific deletion of *Dagla* in neurons, astrocytes or microglia cells were generated, in order to identify the cellular source of 2-AG in the brain and to clarify the role of DAGLa in these cells in affective behaviors.

Using the highly sensitive RNAscope technology, we show for the first time that a subpopulation of astrocytes in the mouse brain expresses *Dagla*, albeit at much lower levels than neurons.

Targeted lipidomics revealed that both, neuronal and astrocytic DAGLa, contribute to the steady-state brain 2-AG levels. However, astrocytic DAGLa only accounts for a small percentage of 2-AG levels and other arachidonic acid derived lipids like prostaglandins, which is in line with the lower expression of DAGLa on astrocytes compared to neurons. Furthermore, our results indicate that deletion of *Dagla* on one of these cell types, can be at least partly compensated by 2-AG production by the other cell types. Thus, we were only able to measure pronounced changes in bulk 2-AG levels in a mouse model with a deletion of *Dagla* in both, astrocytes and some neurons. Under normal conditions *in vivo*, microglial DAGLa does not contribute to bulk brain 2-AG levels. However, it has been shown that microglial cells are main producers of 2-AG in inflammatory conditions.

On the behavioral level, neuronal knockout of DAGLa led to clear changes in anxiety-related behaviors, measured in the zero-maze and open field test. These changes were accompanied with highly elevated corticosterone levels, suggesting an extremely important role of neuronal DAGLa in the regulation of stress and anxiety. Furthermore, mice with a neuronal *Dagla* deletion showed highly decreased body weight and food intake, implicating that neuronal DAGLa controls metabolic functions.

Although astrocytic DAGLa only accounts for a minor percentage of the steady-state brain 2-AG, the deletion of *Dagla* in adult mouse astrocytes had profound behavioral consequences with

significantly increased depressive-like behavioral responses and striking effects on maternal behavior. Our findings therefore indicate that lipids from the DAGLa metabolic axis in astrocytes play a key regulatory role in depressive-like behaviors. The depression-like phenotype was associated with decreased adult hippocampal neurogenesis, suggesting a highly important role of DAGLa in astroglial cells in the regulation of this process, possibly via fibroblast growth factor 2 (FGF2).

Conditional knockout mice that were used to investigate DAGLa in microglia, showed slightly increased anxiety-related behaviors and decreased survival of neural progenitor cells in the dentate gyrus. These phenotypes can rather be attributed to the loss of DAGLa on myeloid cells in the periphery than to DAGLa in microglia, based on the characterization of knockout efficacy in our mouse lines

Altogether, data from this thesis suggests that DAGLa in both, neurons and astrocytes, contributes to the steady-state 2-AG levels in the brain. DAGLa in both cell types is involved in the pathology of affective behaviors, with neuronal DAGLa playing a role in anxiety-related behaviors while DAGLa on astrocytes is especially involved in maternal and depressive-like behaviors. Proliferation and survival of progenitor cells during adult hippocampal neurogenesis are regulated by DAGLa on astroglial cells and myeloid cells, respectively.

Contents

1 Introduction

1.1 The endocannabinoid system

The plant *Cannabis sativa,* better known as marijuana, has been used for medical and spiritual purposes for a long time in human history. First records of its usage in the treatment of cramps and pain, can be traced back around 5000 years ago (Mechoulam 2019). However, the finding that this plant includes more than 100 (phyto)cannabinoids is much more recent (Marcu 2016). The most popular cannabinoid derived from *Cannabis sativa*, (−)-Δ9-trans-Tetrahydrocannabinol (THC), is the major psychoactive component of the plant and was first characterized by Raphael Mechoulam and colleagues in 1964 (Gaoni and Mechoulam 1964).

Besides the plant-derived phytocannabinoids, two main endogenous cannabinoids (endocannabinoids) of the mammalian body have been identified: anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (W. Devane et al. 1992; Mechoulam et al. 1995). Similar to THC, both endocannabinoids are lipid compounds (Figure 1.1). Unlike most other neurotransmitters, endocannabinoids are not stored in vesicles but produced on demand. They are best known to be produced from precursor lipids in the post-synapse and act as retrograde messengers on the pre-synapse, through which they regulate neurotransmitter release (Howlett et al. 2002).

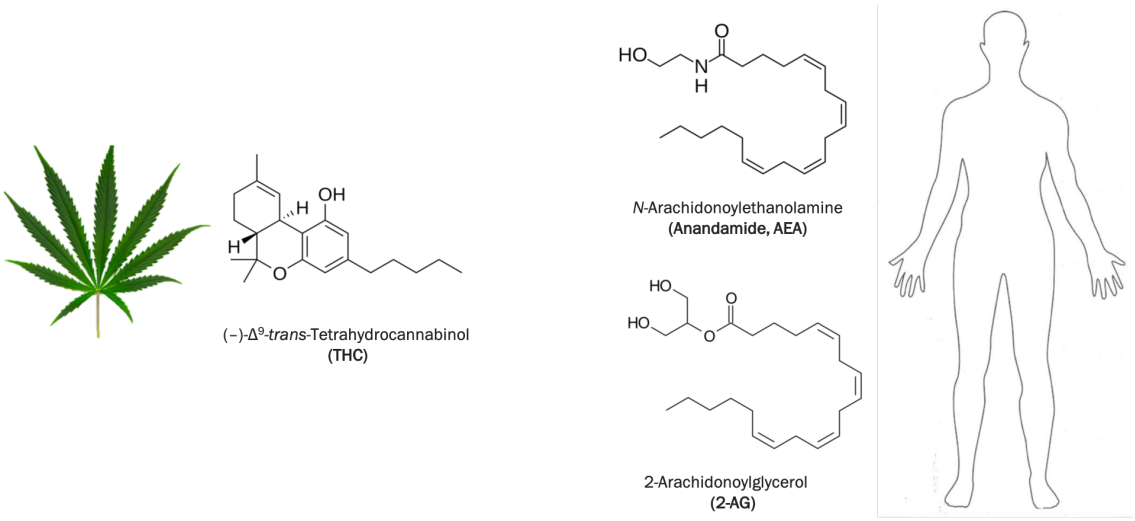


Figure 1.1 Plant-derived and endogenous cannabinoids.

Structural formula of the main psychoactive cannabinoid derived from the plant Cannabis sativa: Δ9-Tetrahydrocannabinol, and the two endogenous cannabinoids: anandamide (AEA) and 2-arachidonoylglycerol (2-AG). (Sources: Cannabis leave: www.pngitem.com; body shape: www.pinterest.de)

Cannabinoids elicit their physiological effects by binding to cannabinoid receptors. The first endogenous cannabinoid receptor, cannabinoid receptor 1 (CB1), was identified in 1988 and cloned in 1990 (W. A. Devane et al. 1988; Matsuda et al. 1990). It is encoded by the gene *Cnr1* and consists of 472 amino acids in humans. CB1 is the most abundant G-protein-coupled receptor (GPCR) in the brain and is found throughout the central nervous system (CNS) and peripheral nervous system (PNS) (Herkenham et al. 1990; Mechoulam and Parker 2013). Its expression within the CNS is particularly high in the hippocampus, cerebellum, basal ganglia, and neocortex, which is in line with the important role of CB1 in cognition and motivation (Eggan and Lewis 2007; Herkenham et al. 1990; Howlett et al. 2002; Mátyás et al. 2006). CB1 can be found on both GABAergic and glutamatergic neurons and is localized pre-synaptically (Howlett et al. 2002; Katona et al. 1999). CB1 was also found to be expressed in astrocytes, oligodendrocytes, microglia and numerous peripheral organs, although at low levels (Castillo et al. 2012; Maccarrone et al. 2015; Stella 2009). Recently, CB1 expression was even found on mitochondria, where it regulates neuronal energy metabolism (Bénard et al. 2012).

Three years after the characterization of CB1, a second cannabinoid receptor CB2, encoded by the gene *Cnr2*, was identified in macrophages of the spleen (S Munro, Thomas, and Abu-Shaar 1993). The two cannabinoid receptors, CB1 and CB2, exhibit 48% amino acid sequence identity. They both belong to the group of 7 transmembrane receptors and are $G_{i/o}$ coupled to adenylyl cyclase and mitogen-activated protein kinase (Mechoulam and Parker 2013). Nevertheless, they show a very distinct expression pattern. CB2 is mainly expressed on immune cells in the periphery and was shown to modulate immune cell functions. In humans, CB2 expression was found in tonsils, spleen, and thymus (Galiègue et al. 1995). Initially, CB2 could not be detected in the brain (Sean Munro, Thomas, and Abu-Shaar 1993). Later on, CB2 was found to be expressed on microglial cells in the brain, especially under inflammatory conditions (P. Pacher and Mechoulam 2011). At the beginning of this century, studies reported CB2 mRNA and protein localization on neurons, but at low levels (Emmanuel S. Onaivi 2007; Emmanuel S. Onaivi et al. 2006).

Additional to the two well-characterized cannabinoid receptors CB1 and CB2, other endocannabinoid targets have been identified. For example, AEA is an endogenous agonist of the “pain receptor” transient receptor potential vanilloid type-1 (TRPV1) and the transient receptor potential cation channel subfamily M (melastatin) member 8 (TRPM8) (Muller, Morales, and Reggio 2019). Furthermore, the orphan G-protein coupled receptors GPR55 (binds AEA & 2-AG) and GPR18 (binds AEA) have been identified as endocannabinoid targets (A. J. Brown 2007; McHugh et al. 2011).

Besides cannabinoids and cannabinoid receptors, the endocannabinoid system comprises of different catabolic and metabolic enzymes. Anandamide is synthesized by the enzymes *N*-acyltransferase (NAT) and *N*-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-

PLD) and is hydrolyzed by the fatty acid amino hydrolase (FAAH). Two diacylglycerol lipases (DAGLa and DAGLb) catalyze the synthesis of 2-AG from diacylglycerol (DAG). 2-AG is metabolized primarily by monoacylglycerol lipase (MAGL).

1.1.1 Diacylglycerol lipases (DAGL) and the synthesis of 2-AG

In the brain, the basal levels of 2-AG are approximately 170 times higher than AEA levels (Stella, Schweitzer, and Plomelli 1997). Furthermore, 2-AG is a full agonist of both cannabinoid receptors with moderate affinity. Even though AEA has a high affinity to cannabinoid receptors, it is only a partial agonist of CB1 and almost inactive at CB2 (Vincenzo Di Marzo and De Petrocellis 2012; Pertwee 2008). Electrophysiological studies have shown that endocannabinoid signaling is almost lost in the hippocampus of mice with reduced 2-AG production (Y. Gao et al. 2010; Tanimura et al. 2010). Considering these facts, 2-AG seems to be the key player in endocannabinoid signaling in the brain. For this reason, this thesis is focused on the endocannabinoid 2-AG, its origin and function.

2-AG is synthesized on demand in the post-synapse. Whether it is also produced by astrocytes and microglia will be disclosed in this thesis. 2-AG acts as a retrograde messenger to inhibit neurotransmitter release from both, excitatory and inhibitory synapses (further information in 1.1.2). 2-AG signaling is associated with many important physiological processes, including homeostasis (Silvestri and Di Marzo 2013), inflammation (Batkai et al. 2011), learning and memory (Griebel et al. 2015), food intake (V. Di Marzo, Ligresti, and Cristino 2009; Vincenzo Di Marzo et al. 2001), locomotor activity (Zimmer et al. 1999), neuroprotection (Van Der Stelt and Di Marzo 2005), reward (Ledent et al. 1999), affective state (Haller et al. 2002), stress and anxiety (Matthew N Hill and Patel 2013; Jenniches et al. 2016a). Due to all those important actions, endocannabinoid signaling is tightly regulated by catalytic and metabolic enzymes which control 2-AG levels (Figure 1.2).

The synthesis of 2-AG starts with two mechanisms. The first is starting from the minor phospholipid of membranes: phosphatidylinositol 4,5-bisphosphate (PIP_2). The second generates diacylglycerol (DAG) from sn2-arachidonate containing triacylglycerols (TAG). In the first pathway, PIP_2 is converted to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) by the enzymes phospholipase C-beta or gamma (PLCß/γ). PLCß uses Ca^{2+} as a cofactor. Thus, the enzyme is activated through signals coming from G_q-coupled receptors or influx of extracellular Ca^{2+} through ionotropic receptors and voltage-gated Ca^{2+} channels. It is also important to note, that DAG, the precursor of 2-AG, serves as an important second messenger signaling lipid, activating protein kinase C. In this thesis, DAG is mostly called 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), which is the precise name of the diacylglycerol involved in the production of 2-AG. Diacylglycerol lipases (DAGL) hydrolyze DAG at the sn1-position into 2-AG. The two existing

isoforms of DAGL, DAGL-alpha (DAGLa) and DAGL-beta (DAGLb), will be further described in 1.1.1.1. An alternative pathway for 2-AG synthesis from PIP_2 has been identified, in which DAGLs are not involved. In this pathway the PIP_2 phosphatase catalyzes the dephosphorylation of PIP_2 generating phosphatidylinositol (PI). Phospholipase A1 is then hydrolyzing the PI to produce 2-arachidonoyl lyso-phosphatidylinositol (2-AG-LPI), which is afterwards converted into 2-AG by the enzyme lyso-phospholipase C (Lyso-PLC) (Nakane et al. 2002). Even though this independent pathway has been identified, DAGLs are still considered the most important enzymes in the biosynthesis of 2-AG, based on studies with DAGLa KO mice (Y. Gao et al. 2010; Jenniches et al. 2016a; Z. Zhang et al. 2015). The pathways that are central to this thesis are marked in black color in Figure 1.2.

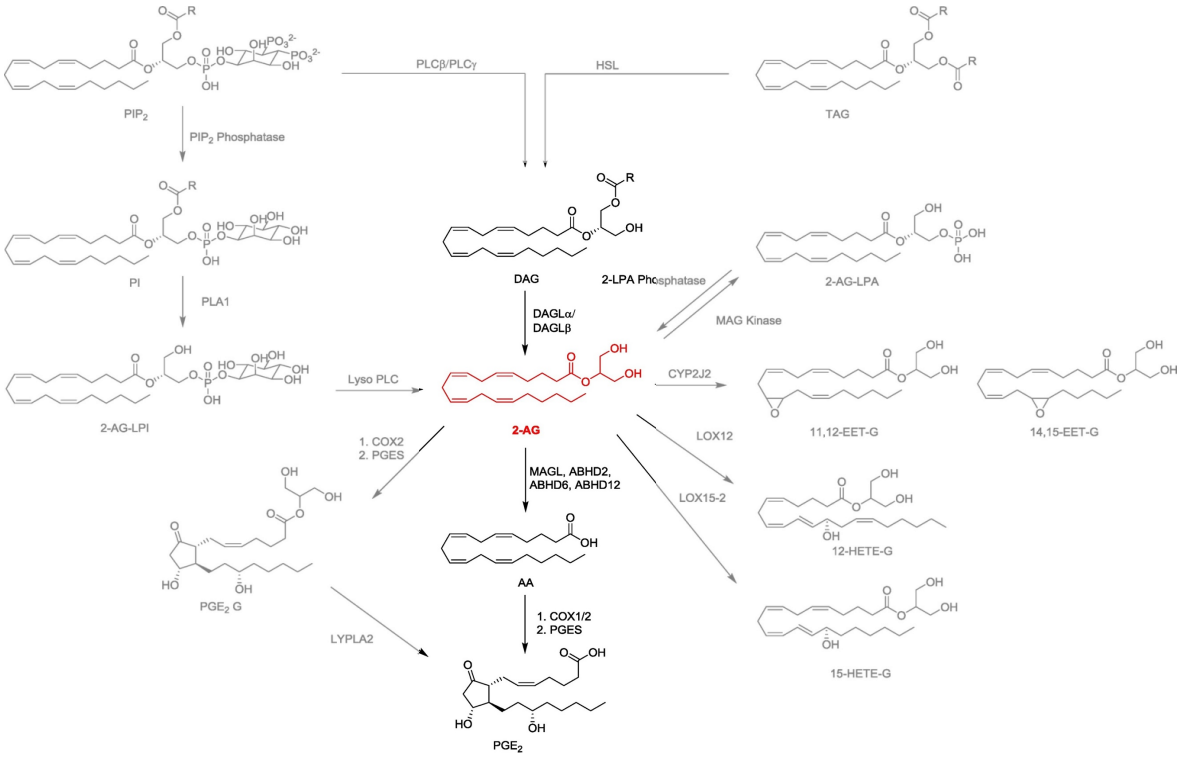


Figure 1.2 Overview of the major biosynthetic and metabolic pathways for 2-AG.

PLCβ: phospholipase Cβ, PLCγ: phospholipase Cγ, HSL: hormone sensitive lipase, PIP2: phosphatidylinositol-4,5-bisphosphate, PI: Phosphatidylinositol, TAG: triacylglycerol, DAG: diacylglycerol, PLA1: phospholipase A1, 2-AG-LPI: 2-arachidonoyl lyso-phosphatidylinositol, Lyso-PLC: lyso-phospholipase C, MAG kinase: monoacylglycerol kinase, 2-LPA phosphatase: 2-lysophospatidic acid phosphatase, 2-AG: 2-arachidonoyl glycerol, 2-AG-LPA: 2-arachidonoylglycerol lysophosphatidic acid, CYP2J2: cytochrome P2J2, LOX-12: lipoxygenase 12, LOX-15-2: lipoxygenase 15-2, MAGL: monoacylglycerol lipase, ABHD2, 6, 12: alpha/beta hydrolase domain containing 2, 6, 12, COX1/2: cyclooxygenase 1/2, LYPLA2: lysophospholipase 2, 11, 12-EET-G: 11, 12-epoxyeicosatrienoic acid glycerol ester, 14, 15-EET-G: 14, 15-epoxyeicosatrienoic acid glycerol ester, PGE2-G: prostaglandin E2 glycerol ester, PGE2: prostaglandin E2, 12-HETE-G: 12-hydroxyeicosatetraenoic glyceryl ester, 15-HETE-G: 15-hydroxyeicosatetraenoic glyceryl ester. PGES: Prostaglandin E synthase. R = predominantly stearoyl, but it can also represent lipids of different chain length and/or degree of saturation (Baggelaar 2018).

1.1.1.1 DAGLa and DAGLb

The two enzymes DAGLa and DAGLb were first identified and cloned in 2003 (Bisogno et al. 2003). The enzymes are encoded on chromosome 11 (*Dagla*) and 7 (*Daglb*) in humans and chromosome 19 (*Dagla*) and 5 (*Daglb*) in mice. They share 97% homology between mice and men for DAGLa and 79% for DAGLb, while the proteins themselves share 33% of sequence identity. DAGLa and DAGLb are plasma membrane-bound proteins containing 4 transmembrane domains. They act as serine-hydrolases specific for the hydrolysis of the acyl chain at the sn1-position of DAG (Bisogno et al. 2003). Comparing both enzymes, DAGLa contains a large C-terminal tail that is not present in DAGLb protein (Figure 1.3). Therefore, DAGLa contains 1042 amino acids and has a molecular weight of ~120 kDa, whereas DAGLb contains only 672 amino acids with a molecular weight of ~70 kDa.

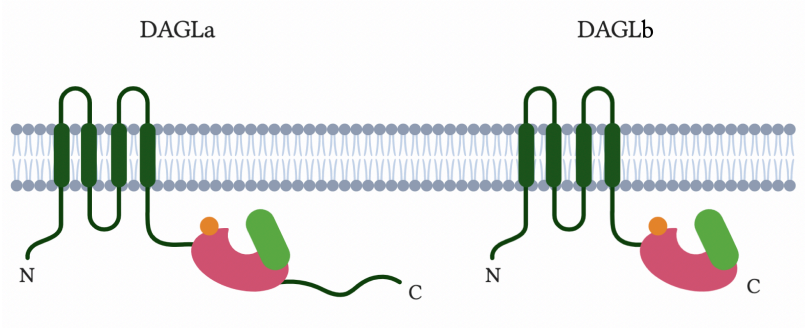


Figure 1.3 Schematic domain structure of DAGLa and DAGLb.

Both enzymes contain four transmembrane domains, a catalytic domain in red, a cysteine rich insert in yellow and regulatory loop in green. In contrast to DAGLb, DAGLa has a long C-terminal tail.

The C-terminal tail of DAGLa is known to have an important regulatory function since phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) on this site significantly reduced enzyme activity (Shonesy et al. 2013). Furthermore, a PPxxF motif on the C terminus can bind to adaptor proteins (Homer proteins), which is crucial for the localization of DAGLa in the plasma membrane close to metabotropic glutamate receptors (Jung et al. 2007). Nevertheless, the catalytic activities of DAGLa and DAGLb do not differ in general (Bisogno et al. 2003). The 1,2,3-Triazole ureas DO34 and DH376 are known inhibitors of DAGLs (Bisogno et al. 2003).

Studies with DAGL knockout mice have shed light on the contribution of DAGLa and DAGLb in the biosynthesis of 2-AG. While a deletion of *Dagla* led to an 80% reduction of 2-AG levels in the brain and spinal cord, *Daglb* KO mice only showed a 50% reduction of 2-AG levels in the brain and almost no reduction in the spinal cord. Furthermore, *Dagla* KO reduced 2-AG levels in the liver about 60% whereas *Daglb* KO mice showed a 90% reduction of 2-AG levels in the liver (Ying Gao et al. 2010). Therefore, the two enzymes seem to have a different contribution to 2-AG

biosynthesis within different tissues, with DAGLa having a more important role in the brain. In the brain, DAGLa is highly expressed in neurons and found mostly at the post-synaptic sites, while DAGLb is more active on microglia (Viader et al. 2016; Yoshida et al. 2006). Whether DAGLa is also expressed on astrocytes and microglia will be disclosed within this thesis.
According to some studies, DAGLa and DAGLb cannot compensate for each other, but there might be a cooperation between both enzymes (Tanimura et al. 2010).

1.1.1.2 Constitutive Dagla knockout mice

Constitutive Dagla KO mice were generated and characterized in our institute (Jenniches et al. 2016a). Those mice showed an 80% reduction of brain 2-AG levels with a concomitant reduction of anadamide levels in the cortex and amygdala. Both male and female DAGLa KO mice showed decreased body weight. *Dagla* deletion led to a reduced exploration in the open-field test, increased distance travelled in the open compartments of the O-maze, a fear extinction deficit and increased anxiety in the dark/light test. Furthermore, mice exhibited increased behavioral despair in the forced swim test and reduced maternal care behavior. Adult neurogenesis was investigated in the subgranular zone of the dentate gyrus in Dagla KO mice. In comparison to control Dagla floxed (Dagla fl/fl) mice, Dagla KO mice showed reduced stem cell/progenitor cell proliferation, estimated using a Bromodeoxyuridine (BrdU) staining (Y. Gao et al. 2010; Jenniches et al. 2016a).

1.1.2 Metabolism of 2-AG

2-AG is can be metabolized by different enzymes (See Figure 1.2). The most prominent player in the metabolism of 2-AG is the enzyme monoacylglycerol lipase (MAGL). It hydrolyzes the ester bond of 2-AG to generate arachidonic acid (AA) and glycerol. MAGL is responsible for 85% of 2-AG metabolism in the mouse brain and is positioned mostly in presynaptic membranes (Blankman, Simon, and Cravatt 2007). MAGL KO mice showed elevated 2-AG levels and exhibited enhanced learning as shown by improved performance in novel object recognition and Morris water maze (Pan et al. 2011).
The enzymes α,β-hydrolase domain-containing protein 12 (ABHD12) and ABHD6 metabolize 9% or 4% of 2-AG, respectively (Blankman, Simon, and Cravatt 2007). ABHD2 was shown to metabolize 2-AG in spermatozoa (M. R. Miller et al. 2016). Besides this predominant pathway, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CytP450) use 2-AG as a substrate (Baggelaar, Maccarrone, and van der Stelt 2018).
There are two existent isoforms of COX, COX1 and COX2, which are both known to be involved in inflammation. For example, COX2 is upregulated in inflammatory states and aspirin serves as

a COX2 inhibitor (Kerola et al. 2009). COX2 oxidizes AA into the pro-inflammatory prostaglandins PGE2 and PGD2. Both PGs are known to promote neuroinflammation (Nomura et al. 2011a). In contrast, 2-AG is known to have anti-inflammatory properties. COX1 is constitutively expressed and prefers 2-AG as a substrate. COX2 shows similar kinetics in metabolizing 2-AG and AA (Kozak, Rowlinson, and Marnett 2000; Rouzer and Marnett 2011). 2-AG is metabolized into prostaglandin glyceryl ester (PGE2-G), which also plays a crucial role in immunomodulation, pain and synaptic plasticity through ERK, MAPK, IP3 and NF-kB pathways (Sang, Zhang, and Chen 2007). PGE2-G can be converted to PGE2 by lysophospholipase A_2 ($LYPLA_2$).

1.1.3 Endocannabinoid signaling

1.1.3.1 Endocannabinoid signaling in neurons

Endocannabinoid signaling in neurons is initiated by the production of 2-AG in the postsynaptic terminals. In contrast to other neurotransmitters, 2-AG is produced on demand and acts mainly as a retrograde messenger. On one hand, 2-AG production can be induced by elevation of intracellular Ca^{2+} levels, caused by the depolarization-induced opening of voltage-gated Ca^{2+} channels. Ca^{2+} elevation in the postsynaptic neuron leads to the activation of Ca^{2+} sensitive enzymes, like phospholipase Cß (PLCß). PLCß uses Ca^{2+} as a co-factor and serves thus as a detector of postsynaptic Ca^{2+}. On the other hand, glutamate release on excitatory neurons activates metabotropic glutamate receptors (mGluR), which in turn activate PLCß (Hashimotodani, Ohno-Shosaku, and Kano 2007; Maejima et al. 2001). Upon activation, PLCß converts membrane PIP_2 to DAG, which is then hydrolyzed to 2-AG by DAGL.

Once synthesized, 2-AG travels retrogradely across the synapse to bind to presynaptic CB1 receptors and induce endocannabinoid signaling. In contrast to polar neurotransmitters that are stored in vesicles, endocannabinoids are neutral lipids that normally tend to associate with membranes. The mechanism, how 2-AG is moving across the synapse and also how the cellular uptake is regulated is still unresolved.

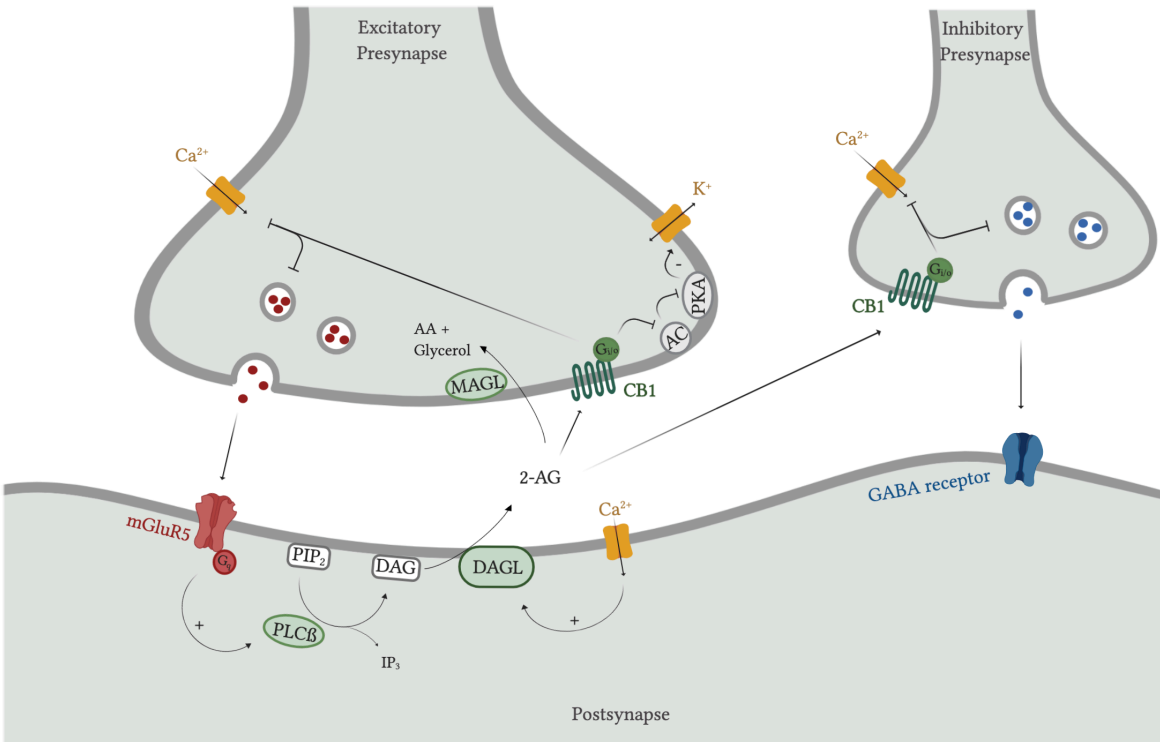


Figure 1.4: Schematic illustration of endocannabinoid signaling in neurons.

2-AG production in the postsynaptic neuron is initiated by activation of metabotropic glutamate receptors (mGluR) or by depolarization-induced increase of intracellular Ca^{2+}. Both mechanisms activate phospholipase C, which in turn converts phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG). DAG is then hydrolyzed to 2-arachidonoylglycerol (2-AG) by diacylglycerol lipase (DAGL). 2-AG acts as retrograde messenger and binds to presynaptic cannabinoid receptors (CB1) to activate endocannabinoid signaling. The G-protein coupled CB1 receptor inhibits Ca^{2+} influx through voltage-gated Ca^{2+}channels and thereby inhibits further neurotransmitter release at the presynaptic neuron. Furthermore, CB1 signaling inhibits the adenylyl cyclase (AC), which causes a downregulation of cyclic adenosine monophosphate (cAMP) and an inhibition of cAMP dependent protein kinase (PKA). Endocannabinoid signaling is terminated by the degradation of 2-AG by the enzyme monoacylglycerol lipase (MAGL) in the presynaptic membrane.

The CB1 receptor is primarily found on central and peripheral neurons in the pre-synapse. CB1 is most abundant in GABAergic interneurons, being between three and ten times less abundant in glutamatergic principal neurons (Kawamura et al. 2006). CB1 are $G_{i/o}$-coupled in neurons and endocannabinoid signaling plays a role in the regulation of synaptic function. CB1 activation through 2-AG inhibits further neurotransmitter release at the pre-synaptic neuron via two mechanisms. For short-time plasticity, CB1 only needs to be activated for a few seconds. Upon activation, the G-protein coupled to CB1 inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels, which in turn inhibits further neurotransmitter release (Figure 1.4) (S. P. Brown, Brenowitz, and Regehr 2003; Kreitzer and Regehr 2001; Wilson, Kunos, and Nicoll 2001). On excitatory glutamatergic synapses, this mechanism is called depolarization-induced suppression of excitation (DSE), while on inhibitory GABAergic synapses it is called depolarization-induced suppression of inhibition (DSI). For long-term plasticity, CB1 activation requires combined presynaptic firing. The $a_{i/o}$ subunit of the G-protein inhibits the adenylyl cyclase, which causes downregulation of cyclic adenosine monophosphate (cAMP) and hence an inhibition of cAMP-

dependent protein kinase (PKA) (Chevaleyre, Takahashi, and Castillo 2006; Heifets and Castillo 2009). Furthermore, CB1 receptor activation leads to a stimulation of mitogen-activated protein kinase (MAPK) activity, affecting synaptic plasticity (or thereby modifying enzymatic activities and gene expression) (Howlett et al. 2002).
Endocannabinoid signaling is terminated by the degradation of 2-AG by MAGL in the presynaptic neuron.

1.1.3.2 Endocannabinoid signaling in astrocytes

Astrocytes are the most abundant glial cells in the nervous system and are about as numerous as neurons. Besides their function in the formation of a blood-brain-barrier, they have a key role in the nutrition of neurons and in keeping a stable homeostatic environment for adequate neuronal function (Magistretti 2006). Moreover, astrocytes exhibit bidirectional communication with neurons, also known as the tripartite synapse (Araque et al. 1999). Indeed, astrocytes are able to detect synaptic signals and modulate neuronal signaling by clearance of neurotransmitters from the synaptic cleft or by releasing gliotransmitters (Araque et al. 1999).
Even though there were some discrepancies about CB1 expression on astrocytes at first, it is now well accepted that brain and spinal cord astrocytes do express CB1, albeit at much lower levels compared to neurons (Hegyi et al. 2009; Marta Navarrete and Araque 2008). Despite the extremely low expression of CB1 in astrocytes, the receptor still has functional relevance. In neurons, CB1 is known to be preferentially coupled to pertussis toxin-sensitive $G_{i/o}$ proteins that regulate cAMP levels (see 1.1.3.1). In contrast, astrocytic CB1 is coupled to $G_{q/11}$ proteins that activate PLC and produce IP_3. Thus, the activation of astroglial CB1 receptors by 2-AG released from pyramidal neurons was shown to mobilize Ca^{2+} from intracellular stores, which in turn triggered the release of the gliotransmitters glutamate and D-serine, and potentiated the transmission in synapses of adjacent neurons (Figure 1.5) (Han et al. 2012a; Marta Navarrete and Araque 2008). At the behavioral level, astroglial CB1 was involved in spatial memory impairments induced by exogenous cannabinoids. This demonstrates that one of the most common side-effect of cannabinoid intoxication by THC is mediated by endocannabinoid signaling in astrocytes (Han et al. 2012a).
In 2018, Hegyi *et al.* showed that cultured rat spinal astrocytes also express DAGLa, the main producing enzyme of 2-AG. They reported that CB1 and DAGLa are located in close vicinity to each other in those astrocytes. Furthermore, there is evidence that evoked Ca^{2+} transients lead to the production of 2-AG in cultured astrocytes (Hegyi et al. 2018).
These findings indicate that astrocytes may possess a fully functional endocannabinoid system, which could be relevant in the regulation of tripartite synaptic plasticity involving astrocytes and neurons.

CB2 does not seem to be expressed in astrocytes.

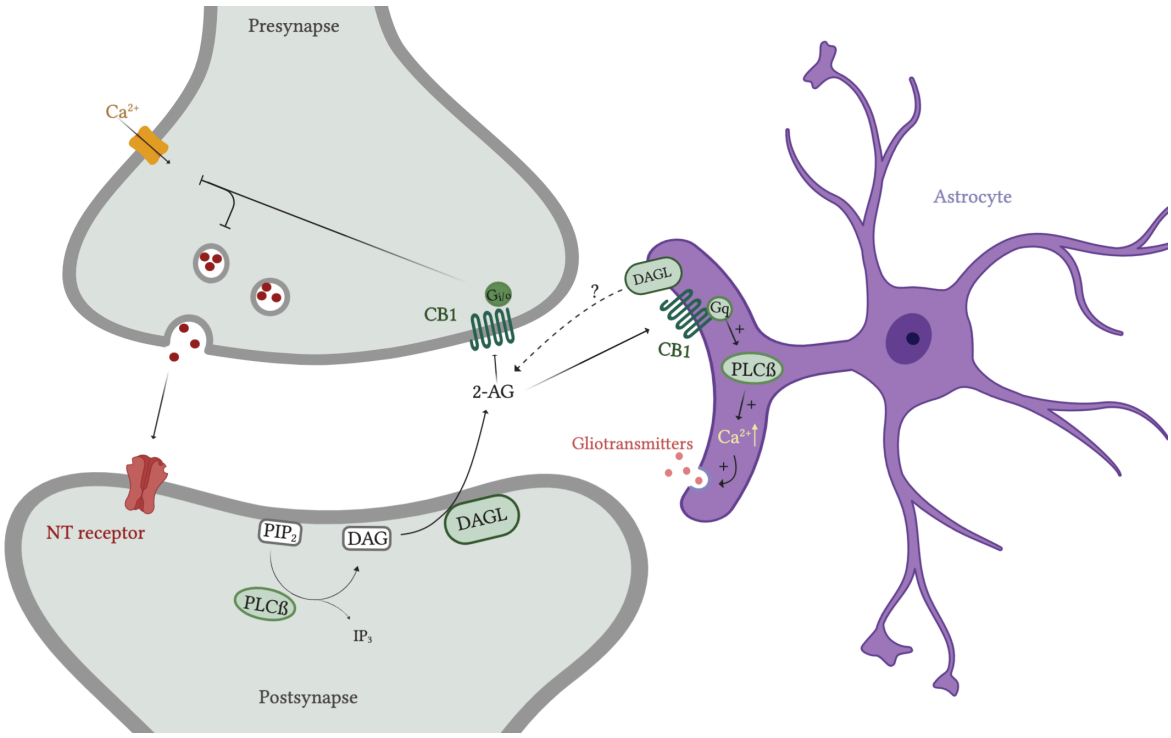


Figure 1.5: Schematic illustration of endocannabinoid signaling in the tripartite synapse.

While CB1 in neuronal terminals is preferentially coupled to $G_{i/o}$ proteins, it is coupled to $G_{q/11}$ proteins in astrocytes. CB1 signaling in astrocytes activates phospholipase C and IP_3 production. This leads to a mobilization of Ca^{2+} from internal stores and the release of gliotransmitters, like glutamate or D-serine.

It is currently unknown, if astrocytes in the brain also produce 2-AG *in situ* and if this contributes to the modulation of behavior.

1.1.3.3 Endocannabinoid signaling in microglia

Microglia cells are the resident immune cells of the CNS, which derive from myeloid progenitor cells during embryogenesis (Ginhoux et al. 2013). The pool of microglia persists throughout life and is maintained by proliferation (Askew et al. 2017). Besides immune functions, microglia also play a key role in brain homeostasis and plasticity by removing redundant synapses, phagocytosis of apoptotic neurons and regulation of neurotransmitter release and neurogenesis (Gomez-Nicola and Perry 2015; Lisboa et al. 2016; Sierra et al. 2010). Under normal conditions in the healthy brain, “resting” microglia constantly scan their environment (Nimmerjahn, Kirchhoff, and Helmchen 2005). Upon brain injury or infection, they undergo morphological changes and aquire an “activated state” (Colton and Wilcock 2012). Thereby, microglia increase their phagocytic activity and start producing pro-inflammatory cytokines and chemokines (Boche, Perry, and Nicoll 2013).

There is not much known about endocannabinoid signaling in microglia cells, especially because most microglia studies were done in cultures. These studies need to be interpreted with caution since microglia adapt a reactive state when cultured (Becher and Antel 1996).

Microglia express both cannabinoid receptors, CB1 and CB2, whereby CB1 is expressed in lower amounts (Stella 2009). In cultured microglia, CB1 was primarily localized in the intracellular compartments and not in the plasma membrane, while expression of CB2 was heterogenous throughout the cells with especially high density in compartments mediating cell migration (Walter et al. 2003). In neurodegenerative disease or inflammatory conditions, expression of CB2 was found to be highly upregulated (up to 200-fold) (Bisogno and Di Marzo 2012; Maresz et al. 2005).

According to a single cell RNA-Sequencing study, DAGLb is much higher expressed in microglia compared to DAGLa (www.brainrnaseq.org). Furthermore, microglial cells in culture produce the endocannabinoids, 2-AG as well as AEA, in smaller amounts (Carrier et al. 2004). The amounts of endocannabinoids produced by microglial cells in culture are approximately 20-fold higher than those produced by neurons or astrocytes in culture, suggesting that microglia cells are the main producers of cannabinoids under inflammatory conditions (Walter et al. 2003). It is also known that activation of P2X7 receptors by ATP, which are highly permeable for Ca^{2+}, is necessary to directly increase DAGLa and reduce MAGL activity and thus increase 2-AG production in microglia (Witting et al. 2004).

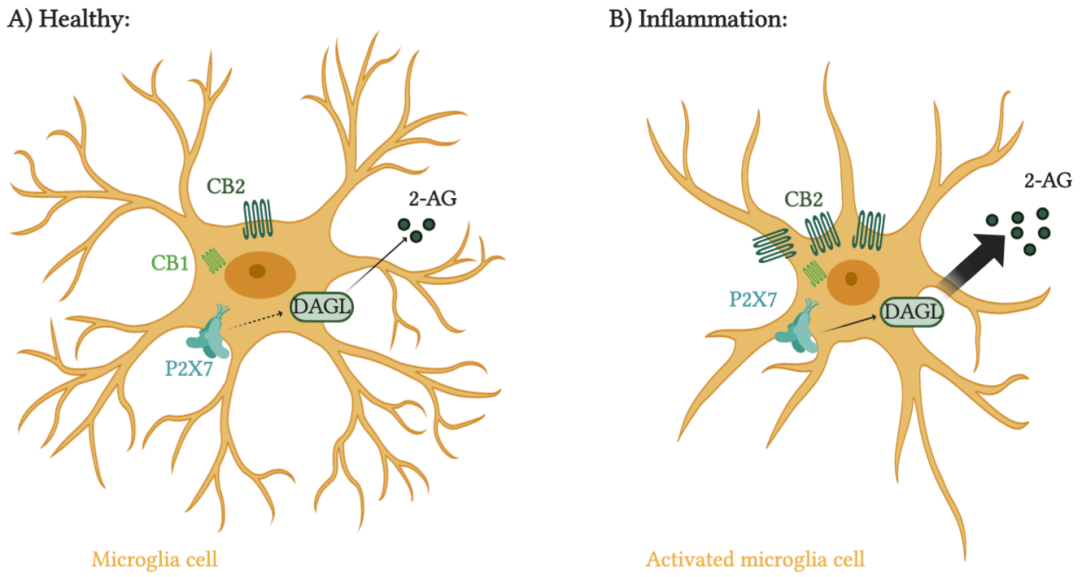


Figure 1.6 Schematic illustration of the endocannabinoid system in microglia cells.

(A) *In the healthy brain, microglia express small amounts of cannabinoid receptor 2 (CB2) and produce small amounts of the endocannabinoid 2-arachidonoylglycerol (2-AG). Cannabinoid receptor 1 (CB1) expression is even lower and CB1 is mainly localized in intracellular compartments. In microglia cells the enzyme diacylglycerol lipase beta (DAGLb) is more expressed than DAGLa.* ***(B)*** *In the diseased brain, microglia overexpress CB2 and produce higher amounts of 2-AG. P2X purinoceptor 7 (P2X7) signaling is necessary for the increased 2-AG production in microglia cells.*

Endocannabinoid signaling in microglia can reduce the production of pro-inflammatory cytokines and increase the production of anti-inflammatory cytokines. Additionally, it can stimulate microglia proliferation and migration (Carrier et al. 2004; Correa et al. 2010; Eljaschewitsch et al. 2006; Walter et al. 2003). Thus, activation of cannabinoid receptors on microglia seems to be involved in migration, proliferation and immune functions. It is not known yet, if microglia produce cannabinoids in the brain under healthy conditions and if cannabinoids produced by microglia cells contribute to normal behavior.

1.1.4 Sex differences in the endocannabinoid system

In humans, exogenous cannabinoids seem to have a greater subjective effect on women, compared to men. For example, women report more dizziness than men having similar plasma THC levels (Mathew, Wilson, and Davis 2003). Furthermore, women tend to become addicted to marijuana faster and relapse more frequently than men (Becker and Hu 2008). Also in rodents, studies showed that females are more sensitive than males to the reinforcing, anxiogenic, spatial memory impairing, hypothermic, antinociceptive and sedative effects of cannabinoids (Cha et al. 2007; Craft et al. 2012; Fattore et al. 2007; Harte and Dow-Edwards 2010; Romero et al. 2002; Tseng and Craft 2001). These sex differences in exogenous cannabinoid effects appear to be strongly influenced by ovarian hormones in adult rodents, especially by estradiol. It was shown, that endocannabinoid levels in female mice are fluctuating during the estrous cycle. The changes in endocannabinoid levels in female rats in comparison to males/OR their baseline levels occurred predominantly in the period surrounding ovulation. During this phase higher levels of 2-AG were measured in the hypothalamus, midbrain, and pituitary, while levels were decreased in the thalamus and hippocampus of female mice (Bradshaw et al. 2006). Another study showed that male rats have higher CB1 levels in the hippocampus than females. However, chronic mild stress produced a downregulation of CB1 expression in the hippocampus of male and upregulation in female rats (Reich, Taylor, and McCarthy 2009).

1.2 The endocannabinoid system in depression and anxiety disorders

Components of the cannabinoid system are abundantly expressed in brain regions involved in emotional processes, like the hippocampus, hypothalamus or amygdala, suggesting a key role of the endocannabinoid system in the regulation of mood (Mackie 2005). Thus, cannabis has been consumed for ages, because of its mood increasing effects. It is therefore not surprising

that dysregulations of the endocannabinoid system have been implicated in several psychiatric disorders. For example, the CB1 antagonist rimonabant, which was released to the market in 2005 to treat obesity, showed strong psychiatric side effects including anxiety and depression. Because of this, it was taken away from the market two years later. However, the underlying neurobiological mechanisms for this effect are not entirely clear.

Preclinical studies in mice with a constitutive *Dagla* deletion showed increased behavioral despair, responsiveness to stress and anxiety-like behavior. The phenotype of these mice was similar to those lacking CB1 (Jenniches et al. 2016b; Shonesy et al. 2014a), thus demonstrating that mice with disrupted endocannabinoid signaling are valid models for depression. Human studies also indicate that genetic variants of endocannabinoid system genes are associated with affective disorders (Hillard and Liu 2014; Stahl et al. 2019). Furthermore, patients suffering from depression show decreased serum endocannabinoid levels and treatment with antidepressants increased 2-AG levels in some brain regions (M. N. Hill et al. 2008; Smaga et al. 2014).

In summary, the endocannabinoid system is an important modulator of emotional processes and the disruption of endocannabinoid signaling leads to the development of psychiatric disorders.

1.2.1 Anxiety disorders

Normal anxiety is defined as an emotional state characterized by an unpleasant feeling of tension and worried thoughts. It involves the expectation of a future threat and is often accompanied by muscular tension, elevated heart rate, restlessness and concentration deficits (American Psychiatric Association (www.psychiatry.org/psychiatrists/practice/dsm)). Evolutionary, feelings of anxiety are not only normal but necessary for survival in potentially dangerous or harmful situations. With an incoming danger, the body reacts by activating the sympathetic nervous system (flight or fight response) and the initiation of an evasive action (Jansen et al. 1995). However, in the case of an anxiety disorder, this emotional state becomes disproportionally intense, unreasonable and/or chronically and negatively impacts patients' quality of life. About 7.3% of people worldwide are affected by an anxiety disorder, with women suffering twice as often as men (American Psychiatric Association 2013; Craske and Stein, 2016). There are multiple types of anxiety disorders with generalized anxiety disorder (GAD), specific phobias, social anxiety disorder, and panic disorder being the most common ones (Craske and Stein 2016). GAD is mainly characterized by excessive out-of-control worrying (Locke, Kirst, and Shultz 2015). In Table 1, different diagnostic criteria for GAD are listed according to the American Psychiatric Association.

Table 1: Diagnostic criteria for generalized anxiety disorder (GAD) according to American Psychiatry Association

Diagnostic Criteria for GAD
1) Excessive anxiety and worry, occurring more days than not for at least 6 months.
2) The individual finds it difficult to control the worry.
3) The anxiety and worry are associated with three (or more) of the following six symptoms:
a. Restlessness or feeling keyed up or on edge.
b. Being easily fatigued.
c. Difficulty concentrating or mind going blank.
d. Irritability.
e. Muscle tension.
f. Sleep disturbance (difficulty falling or staying asleep, or restless, unsatisfying sleep).
4) The anxiety, worry, or physical symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
5) The disturbance is not attributable to the physiological effects of a substance (e.g., a drug of abuse, a medication) or another medical condition (e.g., hyperthyroidism).
6) The disturbance is not better explained by another mental disorder.

There are different treatment options for anxiety disorders, including psychotherapy or pharmacological treatment. In psychotherapy, “cognitive behavioral therapy” is often used, which teaches how to recognize and change thought patterns or behaviors that trigger anxiety or panic (Borkovec et al. 2001). In pharmacological treatment, antidepressant drugs can be efficient in reducing symptoms of anxiety disorders. Often, selective serotonin reuptake inhibitors like escitalopram (Laxapro®) or fluoxetine (Prozac®) are prescribed. They are especially helpful because anxiety disorders are often correlated with major depression disorder (MDD) (Lamers et al. 2011). A second variant of pharmacological treatments is the usage of benzodiazepines (positive allosteric modulators of GABA receptors), like alprazolam (Xanax®), clonazepam (Klonopin®) or diazepam with anxiolytic effects. Because of the relatively high risk of addiction and abuse, benzodiazepines are no longer the drug of choice (Borkovec et al. 2001). One of the newer drugs in the treatment of GAD is Pregabalin. This drug seems to have anxiolytic effects through the binding of voltage-gated calcium channels, resulting in a reduction in neurotransmission in excited neurons similar to the action of endocannabinoids (Micó and Prieto 2012).

1.2.1.1 Involvement of the endocannabinoid system in anxiety disorders

Cannabinoid signaling via CB1 has a key role in the modulation of anxiety-related behaviors. Although cannabis usually elicits anxiolytic effects in most users, there are also many known

cases where users developed intense panic and paranoia in response to cannabis consumption (Szuster, Pontius, and Campos 1988; Thomas 1996). It has also been shown that treatment with CB1 agonists has biphasic effects in preclinical models of anxiety; low agonist doses were leading to an anxiolytic effect (Patel and Hillard 2006) whereas high doses of agonists led to anxiogenic effects (Zanettini et al. 2011). This biphasic effect of cannabinoid signaling on anxiety can be either explained by the different distribution of CB1 receptors on glutamatergic and GABAergic neurons (Bellocchio et al. 2010; Lafenêtre, Chaouloff, and Marsicano 2009), by different expression levels of CB1 in different brain regions or by environmental conditions such as prolonged exposure to stress. For the latter, it has been shown that stressful conditions can shift anxiolytic doses of CB1 receptor agonists to produce anxiogenic responses (Matthew N. Hill and Gorzalka 2004). Intracerebral microinjections have demonstrated that anxiolytic effects are mediated by activation of CB1 in the prefrontal cortex and ventral hippocampus (Lisboa et al. 2008; Rubino et al. 2008); whereas activation of CB1 in the dorsal hippocampus and basolateral amygdala led to anxiogenic effects (E. S. Onaivi et al. 1995; Roohbakhsh et al. 2007; Rubino et al. 2008).

Constitutive genetic deletion or a pharmacological blockade of CB1 by rimonabant led to increased anxiety-like behavior in tests such as the light-dark box, elevated plus maze and social interaction test (Castañé et al. 2002; Haller et al. 2002). Moreover, increased anxiety, impaired fear extinction and increased susceptibility to stress-induced anxiety has been observed in mice lacking DAGLa (Bluett et al. 2017; Cavener et al. 2018; Shonesy et al. 2014b). Increased anxiety could be reversed by normalization of 2-AG levels through a pharmacological blockade of MAGL (Shonesy et al., 2014b). In studies that used the MAGL inhibitor JZL184 to increase 2-AG levels, anxiolytic effects were observed (Aliczki et al. 2013; Long et al. 2009; Sciolino, Zhou, and Hohmann 2011). According to those studies, it seems that 2-AG rather than its downstream metabolites has an anxiety-reducing effect. A very recent study has shown that especially glutamatergic signaling in the basolateral amygdala (BLA) - dorsomedial prefrontal cortex (dmPFC) circuit leads to stress-induced anxiety behavior in mice and that 2-AG is the main component to inhibit/regulate this process (Marcus et al. 2019).

Regarding the role of endocannabinoid signaling in the action of anxiolytic drugs (like benzodiazepines), it has been shown that both 2-AG and AEA levels were elevated in the amygdala after diazepam treatment (Micale et al. 2009), suggesting that endocannabinoids play a role in the function of conventional anxiolytics.

1.2.2 Major depressive disorder (MDD)

Major depressive disorder (MDD) is a common and severe affective disorder characterized by persistent feelings of sadness, worthlessness, feelings of guilt, hopelessness and low

motivation. Often patients suffering from MDD even have cognitive impairments and suicidal thoughts. Depression is not a unitary disease but has very diverse phenotypes. MDD is one of the most prevalent lifetime disorders with 16.6% (DSM-5) and the World Health Organization (WHO) ranks MDD as the leading cause of disability worldwide (World Health Organization, 2017). Women have a two-fold increased risk of MDD compared to men (Van de Velde, Bracke, and Levecque 2010). The American Psychiatric Association outlines the following criteria to be diagnosed with depression in the DSM-5 (Table 2). The individual must be experiencing five or more symptoms during the same two-week period and at least one of the symptoms should be either (1) depressed mood or (2) loss of interest or pleasure.

Table 2: Diagnostic criteria for major depression order (MDD) according to the American Psychiatric Association

Diagnostic Criteria for MDD
1) Depressed mood most of the day, nearly every day.
2) Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day.
3) Significant weight loss when not dieting or weight gain, or decrease or increase in appetite nearly every day.
4) A slowing down of thought and a reduction of physical movement (observable by others, not merely subjective feelings of restlessness or being slowed down).
5) Fatigue or loss of energy nearly every day.
6) Feelings of worthlessness or excessive or inappropriate guilt nearly every day.
7) Diminished ability to think or concentrate, or indecisiveness, nearly every day.
8) Recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

The pathology of depressive disorder is not very well understood. It seems to have a multifactorial etiology involving genetic, biological and environmental factors. The disorder is complex. Therefore only some examples for each factor will be pointed out in this paragraph.
Regarding genetic factors, it has been shown that MDD is heritable in 28-44% of cases (Fernandez-Pujals et al. 2015). Moreover, a monozygotic twin study showed that the genetic component is only one factor in the development of depression, because in less than half of the cases both twins were suffering from depression. Furthermore, the study demonstrated that epigenetic factors play a role in the pathology of depression, because the twin suffering from MDD had a higher variation of DNA methylation than their unaffected co-twin (Byrne et al. 2013). One genetic factor that is thought to be involved in the pathology of MDD is a variation of the promoter regions of the serotonin transporter gene (5-HTTLRP – serotonin-transporter-linked polymorphic region of *SLC6A4*). The length of this promotor region is varying in the population

and it was shown that people with the short gene variation are more sensitive to stress and have a higher risk for depression (Daniele et al. 2011; Karg et al. 2011). Other genes are also possible candidates in the pathology of depression according to a meta-analysis. Examples are polymorphisms in the glucocorticoid receptor gene (*NR3C1*) (Van Rossum et al. 2006), the monoamine oxidase A gene *(MAOA)* (Fan et al. 2010), the group-2 metabotropic glutamate receptor gene (*GRM3*) (Tsunoka et al. 2009), brain-derived neurotrophic factor (*BDNF*) (Licinio, Dong, and Wong 2009) or genetic variation in FKBP5 - a protein that helps to regulate cortisol binding to the glucocorticoid receptor (Binder 2009). (For review see (Kupfer, Frank, and Phillips 2012))

On molecular level, imbalances in the production and transmission of neurotransmitters, such as serotonin, dopamine, noradrenaline, and glutamate are commonly observed in MDD (Maletic et al. 2007). Especially deficiencies in serotonin availability, which was studied on tryptophan depletion models, are known to be involved in depression (Toker et al. 2010). Furthermore, abnormalities affecting serotonin receptor and increased monoamine oxidase have been observed in the brains of depressed patients compared to healthy controls (Carr and Lucki 2011; Meyer et al. 2006). Besides neurotransmitter imbalances, it also seems that inflammation plays a role in depression. In stressed or depressed individuals elevated secretion and production of inflammatory cytokines have been documented (Miller AH and Raison CL 2016) and also some anti-depressants act by normalizing concentrations of cytokines or inhibiting their production (Brustolim et al. 2006).

One of the most prevalent environmental factors involved in the development of depression is stress. First of all, several mouse studies have shown that chronic stress can lead to the development of depressive-like behavior (Burgado et al. 2014; Paul Willner, Muscat, and Papp 1992; Xia et al. 2016). In humans, many studies have been conducted to elucidate the connection between stressful life events and the development of depression. Most of the studies found an association between exposure to stressful life events and subsequent onset of episodes of major depression (Kessler, 2013). Stress is activating the hypothalamic-pituitary-adrenal axis (HPA axis), the most important pathway in stress responses in mammals (Figure 1.7). The HPA-axis works as follows: stress promotes the release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH initiates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary and this promotes the secretion of glucocorticoids from the adrenal glands, which leads to the elevation of glucocorticoid levels in the blood and cerebrospinal fluid. Elevated glucocorticoid levels give negative feedback to glucocorticoid receptors in the hypothalamus and hippocampus to suppress further CRH secretion and to the pituitary gland to reduce ACTH release (Sapolsky, Meaney, and McEwen 1985). Patients suffering from depression commonly show dysregulations in the HPA-axis, characterized by elevated cortisol (main human glucocorticoid) secretion in melancholic depression and reduced levels in atypical depression (Gold and Chrousos 2002; Pariante and Lightman 2008). Also,

hypersecretion of CRH as well as decreased responsiveness to glucocorticoids has been observed in depressed patients (Pariante and Lightman 2008). Another set of evidence for HPA-axis being involved in depression is that patients often show an increased size and hyperactivity of the pituitary and adrenal glands (Nemeroff et al. 1992). Depressive patients also show decreased hippocampal volume, which could also be a side effect of decreased neurogenesis (further described in 1.3.). The weighted average showed a reduction of hippocampal volume of 8% on the left hemisphere and 10% on the right hemisphere measured by MRI (Videbech and Ravnkilde 2004).

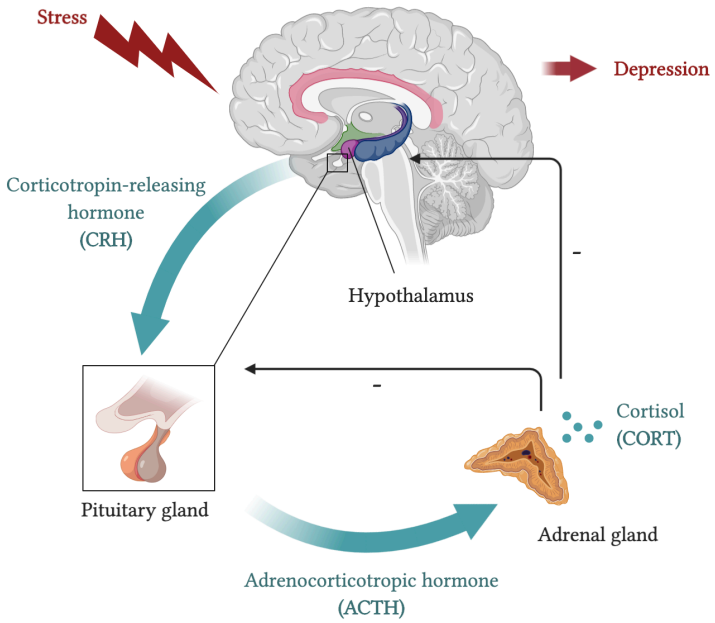


Figure 1.7 Schematic illustration of the hypothalamic–pituitary–adrenal axis (HPA-axis).

Upon stress, the hypothalamus releases corticotropin-releasing hormone (CRH), which stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal glands to produces glucocorticoids (cortisol in humans) which is released into the blood circulation and promotes behavioral effects of stress. Glucocorticoids give negative feedback to the hypothalamus and pituitary gland to reduce the secretion of hormones. Dysregulation in the HPA-axis or chronic stress can lead to the development of anxiety-related or depressive disorders.

The efficient treatment of depression is challenging since the exact pathology of the disorder is unknown. Furthermore, the symptoms of different patients are varying substantially. Psychotherapy and antidepressants are the common treatment options. The treatment with antidepressants is again complicated, because finding the right drug plus the right dosage can be difficult. Often, a combination of different drugs is useful, depending on the symptoms of the patients and the effectiveness of different drugs. It has been shown, that the efficacy of antidepressants depends a lot on the severity of the disorder. For low to middle severity, antidepressants only show low or no effectiveness, while they are more effective in very severe cases (Fournier et al. 2010). In general, the effectiveness of antidepressants is controversial

with several studies showing a similar effect of antidepressants compared to the placebo group (Munkholm, Paludan-Müller, and Boesen 2019) and others demonstrating that up to 50% of the treated patients with severe depression are resulting from the treatment compared to the placebo group (Undurraga and Baldessarini 2012). To find the right dosage of antidepressants to avoid side-effects, a new but quite expensive genetic test has been implicated. This test is evaluating the activity of cytochrome P450 (CYP), an enzyme involved in the metabolism of antidepressants. Furthermore, the test is useful to analyze the absorption efficacy of antidepressants through the blood-brain-barrier (C. Rosenhagen and Uhr 2012; O'Brien et al. 2012).

The first developed group of antidepressants was tricyclic antidepressants (TCA) which act by inhibition of synaptic reuptake of norepinephrine (NE) and serotonin (5-HT) (Racagni and Popoli 2008). This group of drugs effectively increases mood but is associated with the occurrence of severe side effects, especially affecting the cardiovascular system. The side effects mainly result from the action of TCA on sodium channels and its effects on the cholinergic and histaminergic receptors and are therefore very dangerous in the case of overdosing (Review Wyska, 2019). In the 1950s, monoamine oxidase inhibitors (MAOIs) were first used in the treatment of MDD. This group of drugs is inhibiting the enzyme involved in the metabolism of monoamines, like NE, 5-HT or dopamine. Unfortunately, those drugs were shown to have many drug-drug interactions. Furthermore, patients using this group of drugs need to follow a strict tyramine-free diet, an amine that is included for example in nuts and cheese (Cipriani et al. 2018). Both TCAs and MAOIs are rarely used anymore because of their low tolerability and safety (Malhi et al. 2015). The most frequently prescribed medications in the treatment of MDD are nowadays selective serotonin reuptake inhibitors (SSRIs), like fluoxetine, and serotonin-norepinephrine reuptake inhibitors (SNRIs) (National Alliance of mental illness (www.nami.org)). Those drugs show similar efficacy and fewer side-effects on the cardiovascular system but can cause gastrointestinal, cognitive and especially sexual impairments (Brambilla et al. 2005). Many similar drugs, all based on interaction with the noradrenergic and/or serotonergic system have been developed, like 5-HT modulators and stimulators (SMS), 5-HT antagonist and reuptake inhibitors (SARIs), NE reuptake inhibitors (NARIs). All of them have the disadvantage of a very late onset of action and it is evident that the antidepressant effect is highly variable. Although SSRIs act very quickly as inhibitors of the reuptake of 5-HT, the maximal antidepressant effect is observed the earliest after two to three weeks of chronic treatment (Fritze, Spanagel, and Noori 2017). The late onset of the drug suggests that the "immediate" pharmacological mechanism is only the beginning for much more complex, and yet unclear, set of adaptive body responses that finally produce the desired effect. These are currently believed to be second messenger pathways and transcription factors (CREB, BDNF), producing changes in neurotransmitter receptor densities and/or adult neurogenesis (Taylor et al. 2005).

Recently, the high-affinity noncompetitive N-methyl-D-aspartate receptor (NMDAR) antagonist ketamine has been found to be useful to treat depressive symptoms in patients that do not respond to other antidepressants. In contrast to other antidepressants, a single subanesthetic dose (0.5 mg/kg) induces rapid (three-four hours) and sustained antidepressant effects in severely depressed patients. It has been shown that a low dose of ketamine increases glutamate activity in the PFC and that it is even catalyzing synaptogenesis and enhanced synaptic functioning through a cascade of molecular events (probably involving mTOR and BDNF), that are not fully understood yet (Hashimoto 2011; N. Li et al. 2011). However, ketamine has a high potential for abuse and is therefore only prescribed for people with severe depression that do not respond to any other treatment. Furthermore, ketamine users show the highest relapse rate (Berman et al. 2000).
Electroconvulsive therapy (ECT) or transcranial magnetic stimulation (TMS) have also been shown to be efficient in the treatment of depression (Freeman, Basson, and Crighton 1978; George et al. 1995).

1.2.2.1 Involvement of the endocannabinoid system in major depression disorder

Preclinical studies also showed a connection of the endocannabinoid system and depression. For instance, mice lacking CB1 displayed an increased depressive-like phenotype in the forced swim test as well as the tail suspension test (Aso et al. 2008; Steiner et al. 2008). Moreover, CB1 KO mice showed stress-induced anhedonia demonstrating that loss of CB1 promoted the development of depressive-like behavior (M.N. Hill and Gorzalka 2009). In line with this, CB1 deficient mice also showed well known biochemical parameters of depression, like changes in CREB (cAMP response element-binding protein) in specific brain areas, lower levels of markers of neuroplasticity and reduced adult neurogenesis (Jin et al. 2004; Rubino et al. 2008, 2009). Since stress is one of the major risk factors for the development of depression, it is also important to note that CB1 is expressed everywhere in the HPA-axis and has important regulatory functions in stress. CB1 KO mice are much more sensitive to stress (Beins et al., 2020; Molecular Psychiatry (in revision)). Studies have generally shown a bi-directional effect of stress on endocannabinoid levels, AEA levels decreasing and 2-AG levels increasing compared to controls (Morena et al. 2016; Patel and Hillard 2008) Furthermore, pharmacological blockade of CB1 by rimonabant induced a depression-like phenotype in rats, similar to the observations that were made in humans treated with rimonabant against obesity (Beyer et al. 2010; Nissen et al. 2008). Deficient endocannabinoid signaling might also be involved in the development of depressive disorders since reduction in circulating endocannabinoids have been documented in women suffering from depression (M. N. Hill et al. 2008). According to Juhasz et al., 2009, genetic variations in the CB1 receptor function can even influence the risk of developing

depression in humans in response to stressful life events. Moreover, a significant increase in CB1 density has been reported in the PFC of depressed suicide victims (Hungund et al. 2004). Cannabinoid signaling induced by exogenous cannabinoids seems to also have antidepressant effects. It is known from epidemiological studies that cannabis users in general display less depressed mood than non-users (M. N. Hill et al. 2008). Moreover, the consumption of cannabis has an antidepressant effect in individuals with mood disorders (Gruber, Jr., and M.E. 1996). The administration of CB1 agonists has similar potency in eliciting antidepressant effects under acute and chronic conditions (Bambico et al. 2007; Matthew N. Hill and Gorzalka 2005; Morrish et al. 2009). This antidepressant effect is very likely mediated by CB1 signaling in the PFC or hippocampus since local injections of agonists in these areas had similar antidepressant effects (Bambico et al. 2007; McLaughlin et al. 2007). Consistently, inhibition of endocannabinoid reuptake produced antidepressant effects in the forced swim, tail suspension and sucrose-preference tests (Adamczyk et al. 2008; Gobbi et al. 2005; Matthew N. Hill and Gorzalka 2005). An antidepressant profile was also induced by elevated AEA levels in FAAH-deficient mice (Cravatt et al. 2001). Furthermore, CB1 signaling promotes adult neurogenesis, a process highly connected with depression (see 1.3; Aguado et al., 2005; Jiang et al., 2005), suggesting that an increase of endocannabinoids or CB1 signaling elicit both, behavioral and biochemical changes that are characteristics for antidepressant agents. TCA like imipramine lead to an upregulation of CB1 in the hippocampus, hypothalamus, and amygdala (Matthew N. Hill et al. 2006, 2008). Both voluntary exercise or sleep deprivation, which are efficient treatments of depression, also lead to increased endocannabinoid levels and CB1 expression in the hippocampus (C. Chen and Bazan 2005; Matthew N. Hill et al. 2010). This data suggests that the endocannabinoid system even plays a role in conventional treatments used for depression.

1.2.3 Involvement of glia cells in affective disorders

Astrocytes are highly heterogenous glia cells and primarily responsible for homeostasis in the CNS. They are the only cells in the brain capable of synthesizing glutamate from glucose and are essential for the supply of neurons with glutamate and GABA. Imbalances of those neurotransmitters are highly involved in depression and therefore astrocytes seem to play a major role in the disorder (Rose, A., and V. 2013). Dysfunctions of astrocytes are highly correlated with MDD. Post-mortem brains of patients with MDD show decreased number or density of astrocytes, deregulation of astrocytic markers and astrocytic hypertrophy (Cotter et al. 2002; Nagy et al. 2015; Torres-Platas et al. 2011). Morphological and numerical alterations in astrocytes have also been frequently observed in animal models of depression (Rajkowska et al. 2013; Sanacora and Banasr 2013). Furthermore, pharmacological inhibition of astroglial gap-junction connectivity resulted in anhedonia, one of the key symptoms of depression (J. D.

Sun et al. 2012). It has also been shown that some antidepressants elicit their effects through astrocytes, since a high production of the fibroblast-growth-factor 2 (FGF-2), a factor involved in synaptogenesis and neurogenesis, has been observed in astrocyte cultures treated with antidepressants but not in neuronal cultures (Kajitani et al. 2012). Astrocytes do express monoamine transporters and the antidepressant fluoxetine triggers an increase of Ca^{2+} in cultured astrocytes (Hertz et al. 2012) as well as astrocytes in brain slices (Schipke, Heuser, and Peters 2011) that might be involved in the action of this drug.

1.3 Adult neurogenesis

Adult neurogenesis is a process in which new neurons are continuously generated in the mammalian adult brain (Altman and Das 1965). First, the process of neurogenesis was thought to only occur during embryonal development and that the structural composition of the neurons within the brain remains unchanged after birth since neurons do not divide. Later on, neural stem cells (NSCs) -self-renewing and multipotent progenitor cells- were also identified in some niches of the adult brain, generating new neurons throughout lifetime at low levels (Altman and Das 1965; Eriksson et al. 1998; Kaplan and Hinds 1977). This finding suggested that the adult brain exhibits more plasticity than previously thought. Erikson was the first to report adult neurogenesis in the hippocampus of the human brain by BrdU labeling in the brains of cancer patients. Since the hippocampus is strongly associated with learning and memory processes, adult neurogenesis has been associated with human cognition. The finding that adult neurogenesis does exist, changed our understanding about how the hippocampus works, regarding human cognition, cognitive aging, and the loss of hippocampal functions in, for example, Alzheimer's disease or stress-related disorders like depression. Recently, the existence of adult neurogenesis in the hippocampus of humans has been debated (Sorrells et al. 2018) but most of the researchers are supporting its existence, pointing out some technical issues in the study by Sorrel *et al.* (Boldrini et al. 2018; Kempermann et al. 2018). In the process of adult neurogenesis, neural stem cells, located in "neurogenic niches" of the brain, can transition between activated and quiescent states by entering and exiting the cell cycle, respectively. Once activated, they can either undergo asymmetric division, which is a self-renewing process and yields an NSC and a neural progenitor, or they undergo symmetric division that yields either two NSCs (self-renewing) or two progenitors (Figure 1.8). These progenitor cells may be multipotent or cell-line restricted and can only differentiate into a particular cell type. It has been postulated for a long time from cell culture studies (high concentration of growth factors) that NSCs are tri-potent cells generating neurons, astrocytes and oligodendrocytes (Palmer, Takahashi, and Gage 1997). However, fate-mapping studies have shown that differentiation is dependent on the neurogenic niche the stem cell reside. In the adult

hippocampus, NSCs were found to generate neurons and astrocytes, but not oligodendrocytes, while SVZ was rather generating neurons and oligodendrocytes (Bonaguidi et al. 2011).

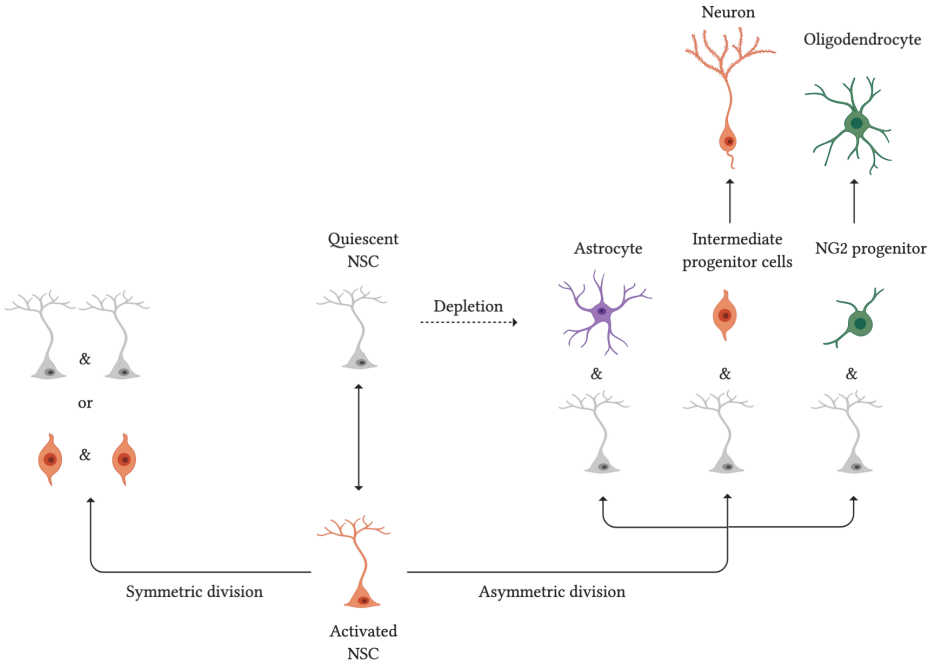


Figure 1.8: Schematic illustration of neural stem cell (NCS) fate in adult neurogenic niches.

NCS can transition between activated and quiescent state. In activated state they can undergo symmetric division to produce either two NSC (self-renewing) or two progenitor cells (orange). Alternatively, they undergo asymmetric division to produce an NCS and a progenitor cell. It is also possible that NSC directly differentiate into mature glia cell type. Figure based on Bond et al., 2015.

There are two major neurogenic niches in the adult mammalian brain, where NSCs reside: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ), along the lateral ventricular walls. Recent studies also showed evidence for adult neurogenesis in the ventral hypothalamus, around the third ventricle, being involved in homeostatic processes and the regulation of food intake (Cheng 2013). Most recently, the striatum was also found to be a neurogenic niche (Ernst et al. 2014) as well as the substantia nigra (M. Zhao et al. 2003) albeit proliferating at much lower levels compared to SGZ and SVZ. Adult neurogenic niches have specific molecular and cellular characteristics that tightly regulate the neuronal development of adult NSCs. They contain ependymal cells, vascular cells and NSC of different maturity (D. K. Ma, Ming, and Song 2005). Unique to SVZ and hypothalamus, ependymal cells (tanycytes in the hypothalamus) are lining the ventricle surface connecting the neurogenic niches to the cerebral spinal fluid (CSF) (Mirzadeh et al. 2008). Ependymal cells secrete signaling factors like noggin, which allow activation of the NSCs. Compared to non-neurogenic niches, the vascular organization in SVZ and SGZ is unique as well, with processes of adult NSC closely associated with blood vessels (Shen et al. 2008; G. J. Sun et al. 2015). Endothelial cells and factors transported through the blood or CSF can provide signals that

impact the NSCs. For example, endothelial cells produce vascular and endothelial growth factor (VEGF) or BDNF which promote adult neurogenesis (Q. Li et al. 2006). Moreover, the blood-brain-barrier is leakier in areas containing NSCs to allow these cells to access factors from the blood more easily (Tavazoie et al. 2008). The extracellular matrix provides a structural framework for NSC in neurogenic niches and is also involved in signaling (Porcheri, Suter, and Jessberger 2014). NSC are embedded in the extracellular matrix and express cell adhesion molecules, like vascular cell adhesion molecule-1 (VCAM-1), that allow them to interact with those molecules of the extracellular matrix that for instance can trap growth factors (like FGF2) (Kerever et al. 2007).

Even though the biological role of adult neurogenesis is far from being fully elucidated, it is now well established that new adult-born neurons from SVZ and SGZ are functionally incorporated in the olfactory bulb or hippocampus, respectively, participating in the function of this areas (Bond, Ming, and Song 2015). In the SVZ, adult NSCs reside along the lateral ventricular walls in a largely quiescent state. Once activated by niche-derived and/or intrinsic signals they undergo proliferation and give rise to transient amplifying cells. Those differentiate into neuroblasts and migrate with each other in chains through the rostral migratory stream (RMS) to the olfactory bulb. Here, neurogenesis continues as the cells differentiate into distinct types of olfactory neurons (mainly inhibitory interneurons) and functionally integrate into the existing circuit. This ongoing adult neurogenesis is essential for maintaining olfactory sensory function (Bond, Ming, and Song 2015).

In the SGZ of the hippocampus, NSCs differentiate into immature neurons and then they migrate into the granule cell layer of dentate gyrus where they mature into excitatory granule cells. These cells then form appropriate synaptic connections. The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibers. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back projections to neurons in the deeper layers of the entorhinal cortex.

In the human brain, approximately 700 new neurons are generated in the hippocampus per day (Spalding et al. 2013). Newly generated neurons in the hippocampus may have functional significance, demonstrated in rodent studies in which hippocampus-dependent behaviors activated newly generated granule cells more often than older cells (Kee et al. 2007; Ramirez-Amaya et al. 2006; Snyder, Choe, et al. 2009; Snyder, Radik, et al. 2009), suggesting that adult hippocampal neurogenesis is important in maintaining hippocampal functions.

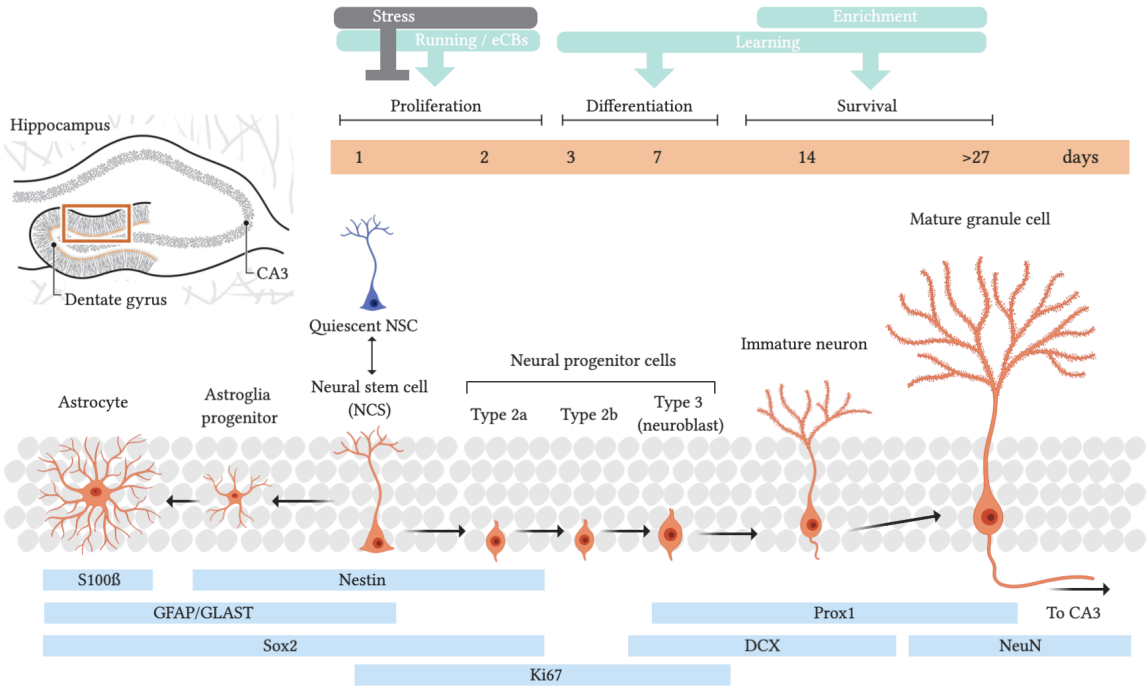


Figure 1.9 Adult neurogenesis in the subgranular zone of dentate gyrus in the hippocampus.

Neural stem cells (NSCs) in the dentate gyrus subgranular zone (SGZ) can transition between activated and a quiescent state. Once activated they can undergo asymmetric division to form neural progenitor cells or astrocytes. Neural progenitor cells can differentiate into immature neurons and migrate into the granule cell layer of dentate gyrus where they mature into excitatory granule cells. Mature granule cells in the dentate gyrus send projections to cornu ammonis 3 (CA3The lower part of the figure shows different markers for the different cell types. In the top of the figure the timeline of the process is shown in days. Adult neurogenesis can be negatively affected by stress and can be promoted by running, endocannabinoids (eCBs), learning or enrichment. Figure based on Gage, 2006.

The generation of functional new neurons is mechanistically a very complex and highly coordinated process, involving proliferation, differentiation, survival, and migration of the NSCs, as well as synaptic integration of the newborn neurons in the existing circuitries. Therefore, adult neurogenesis is highly regulated by different intrinsic factors like transcription factors (e.g. Sox2, NeuroD, Ascl1), intrinsic signaling pathways (Notch, Wnt, and sonic hedgehog) (Hitoshi et al. 2002) and extrinsic factors (e.g. neurotrophic BDNF and NGF) (Birch and Kelly 2013; Scharfman et al. 2005), growth factors (e.g. VEGF, FGF) (Ever et al. 2008; Jin et al. 2002), hormones (e.g. thyroxin, glucocorticoids), cytokines, and neurotransmitters (Bolteus and Bordey 2004; Cameron, McEwen, and Gould 1995). Furthermore, both exogenous and endogenous cannabinoids can control cell genesis in the adult brain (see 1.3.2) (Andersen et al. 2014). Adult neurogenesis is also impacted by different environmental conditions such as aging, stress, inflammation, exercise or antidepressants (Dranovsky et al. 2011; Gould et al. 1997; Malberg et al. 2000; Van Praag et al. 2005).

Abnormalities in adult neurogenesis are associated with neurodegenerative disorders or affective disorders like anxiety and depression as is explained in the next section.

1.3.1 Adult neurogenesis in depression

Most antidepressants act primarily through the monoamine system (see 1.2.2). However, the downstream mechanisms by which they improve mood are still not entirely understood. It is documented that antidepressant treatment increases the number of newborn neurons in the hippocampus of rodents as well as humans (Boldrini et al. 2009; Malberg et al. 2000). The generation of new neurons takes about four weeks, which is similar to the time when antidepressants elicit their effects (Duman, Nakagawa, and Malberg 2001; Kee et al. 2007). A pioneering study, using genetic and radiological methods, described that disrupting antidepressant-induced neurogenesis in the hippocampus blocks the behavioral responses to antidepressants (Santarelli et al. 2003).

To investigate if increasing adult neurogenesis is sufficient for antidepressant-like effect on behavior, several studies were done. In one of them, genetic deletion of neurofibromin, a tumor suppressor with RAS-GAP activity, was used to increase neurogenesis in DG of mice, which had antidepressant effects on behavior in forced swim and tail suspension test (8 month old mice) and mice showed enhanced sensitivity to antidepressants (Y. Li et al. 2012). Another study was using conditional Nestin-Bax KO mice, with a progenitor-specific knockout of Bax (cofactor of tumor suppressor p53) in progenitor cells to increase adult neurogenesis. This study did not find effects on anxiety or depression-like behavior in mice under normal conditions but it reduced anxiety- and depression-like behavior in mice chronically treated with corticosterone (A. S. Hill, Sahay, and Hen 2015). Some other studies were investigating the compound P7C3, a drug with pro-neurogenic effects, which has also been shown to increase adult neurogenesis. Those studies found antidepressant effects of this compound on social interaction following social defeat. However, it is likely that this compound did not only affect neurogenesis (Walker et al. 2015; G. Wang et al. 2014).

It was also shown that neurogenesis and neuronal plasticity are impaired in depression, leading to subsequent neurodegeneration. This results in alterations in the number and morphology of glial cells and neurons in brain regions of depressed patients (Duman 2009; Eyre and Baune 2012). BDNF, an abundant and widely distributed neurotrophin in the CNS, regulating neuronal survival, growth, and proliferation, was also found to be decreased in depressive patients (Lee and Kim 2010). Similarly, the expression of the neurotrophic fibroblast growth factor 2 (FGF2) was found to be decreased in PFC of depressive patients (Evans et al. 2004).

Nevertheless, it is debated if decreased adult neurogenesis directly results in the development of depressive symptoms or if decreased number of neurons in the hippocampus is a consequence of the disorder.

1.3.2 Adult neurogenesis and the endocannabinoid system

Endocannabinoid signaling has a regulatory role in both embryonic and adult neurogenesis (De Oliveira et al. 2019; Prenderville, Kelly, and Downer 2015). Embryonic and adult neural stem cells and progenitor cells express a functional endocannabinoid system (Aguado et al. 2005a). Goncalves *et al.* showed that neural stem cells in the SVZ do express DAGLa and that DAGLa is highly involved in the regulation of adult neurogenesis (Goncalves et al. 2008). While CB1 is generally present at higher levels than CB2 in NSCs (Compagnucci et al. 2013), the expression of CB1 and CB2 in NSCs and their descendant neurons are changing during neuronal development: CB1 levels increase with maturation, whereas CB2 levels are more abundant in immature cells. Endocannabinoid signaling in NSC modulates a variety of cellular processes like proliferation, survival, differentiation, migration, and survival mainly due to activation of ERK1/2; p38 MAPK/JNKs and PI3K/Akt/mTOR pathways (Galve-Roperh et al. 2013). This is supported by the fact that CB1-, CB2-, Dagla- and Daglb-KO mice show reduced adult neurogenesis (Jin et al., 2004; Aguado et al., 2005; Jenniches et al., 2016a; Gao et al., 2010a) In adult neurogenesis, CB1 signaling seems to have a pro-neurogenic role under both normal and pathological conditions. For instance, CB1 stimulation by ACEA, a selective CB1 agonist, had a strong impact on proliferation, differentiation, and migration in the hippocampus of a mouse model of epilepsy (Andres-Mach et al. 2017). Furthermore, the chronic administration of 2-AG ether was able to promote neurogenesis in the mouse olfactory bulb, which could be blocked by the CB1 antagonist AM251 (Hutch and Hegg 2016). In a rat model of alcohol-intoxication, ACEA could reverse impairments in neurogenesis induced by alcohol in the SGZ but not in the SVZ (Rivera, Blanco, et al. 2015). *In vitro* studies demonstrated that ACEA enhanced survival of human NSCs and promoted the proliferation of SVZ-derived rat NSCs. In contrast, ACEA alone was not sufficient to stimulate proliferation of SGZ-derived NSCs, however, it did so in combination with HU308 (CB2 agonist). Also, a co-stimulation of both cannabinoid receptors by WIN55, increased neurogenesis in the SGZ but not in SVZ (Table 3) (Rodrigues et al. 2017).
CB2 signaling has a more prominent role in the regulation of adult neurogenesis under pathological conditions by protecting new neurons from excitotoxicity, oxidative and inflammatory damage. For instance, chronic treatment with the CB2 agonist AM1241 prevented deficits in SGZ neurogenesis in a model of HIV-1- associated encephalitis (Avraham et al. 2014). Moreover, AM1241 increased the survival of neurons and stimulated adult neurogenesis in the substantia nigra in a mouse model of Parkinson's disease (Shi et al. 2017). Several other pathologies in which CB2 signaling enhanced neurogenesis have been identified (Bravo-Ferrer et al. 2017; Rivera, Blanco, et al. 2015; Wu et al. 2017). CB2-dependent effects seem to involve PI3K/Akt/Mek pathways and enhance expression of proteins involved in mitochondrial quality control (Shi et al. 2017).

Table 3: Effects of the cannabinoid system on adult neurogenesis

Treatment	Measurement	Observation	Reference
THC/CBD	Precursor cell proliferation in DG	reduced	Wolf et al., 2010
CBD	Cell survival in DG	enhanced	Wolf et al., 2010
CBD	Number of new neurons in DG	enhanced	Campos et al., 2013
HU-308 (Agonist)	Progenitor proliferation in DG	enhanced	Palazuelos et al., 2012
WIN55 (Agonist)	Proliferation in SGZ, but not SVZ Neuronal differentiation in SVZ and SGZ	enhanced enhanced	Rodrigues et al., 2017
DAGL inhibitor	Cell proliferation in adult SVZ	reduced	Goncalves et al., 2008
DAGLa KO	Cell proliferation and number of new neurons in hippocampus	reduced	Gao et al.2010
DAGLb KO	Cell proliferation in hippocampus	reduced	Gao et al., 2010
CB1 KO	Cell proliferation in SGZ and SVZ	reduced	Jin et al., 2004; Kim et al., 2006
CB2 KO	Proliferation in SGZ	reduced	Palazuelos et al., 2006

Studies using the FAAH inhibitor URB597 or the MAGL inhibitor JZL184 to increase AEA or 2-AG levels respectively, in order to increase adult neurogenesis, have led to conflicting results. Earlier studies suggested that FAAH inhibition enhanced AEA and thereby promoted adult neurogenesis (Goncalves et al. 2008), whereas more recent studies even showed reductions in adult neurogenesis in SGZ after chronic treatment with above mentioned drugs (Aguado et al. 2005a). In contrast, acute URB597 administration led to increased proliferation in SVZ (Rivera, Bindila, et al. 2015). A co-administration of both, JZL184 and URB597 efficiently increased adult neurogenesis in the SVZ (Z. Zhang et al. 2015). Inhibition of MAGL prevented impairments in neurogenesis in the hippocampus of stressed mice (Zhong et al. 2014).

1.4 Aims of the thesis

The endocannabinoid system has been linked to several psychiatric disorders, including anxiety and depression. Since endocannabinoids are produced on demand, their concentrations in the brain need to be tightly regulated by synthesizing and metabolic enzymes. Constitutive genetic deletion of DAGLa, the main producing enzyme of the endocannabinoid 2-AG, has been shown to lead to the development of anxiety-related and depression-like behaviors and to highly decreased 2-AG levels in the brain. However, the cellular source of 2-AG in the CNS is unknown to date.

Thus, the **aim of this study was to investigate the role DAGLa in specific cell types** and to find the main cellular source of 2-AG in the brain. Therefore, conditional DAGLa KO mice with a cell-specific deletion of DAGLa in **neurons, astrocytes and microglia** were used. More specifically, this study was performed to **clarify the role of DAGLa in different cell types in the context of anxiety and depression-like behavior.** Since psychiatric disorders, especially depression, are often accompanied by changes in adult neurogenesis, another goal was to **identify the cell types responsible for the regulation of adult hippocampal neurogenesis by producing endocannabinoids**.

2 Material and Methods

2.1 Equipment

Technical instrument	Identifier, Company
Analytical balance	BP 121 S, Sartorius
Animal tracking software	EthoVision® XT, Noldus
Cell culture incubator	Binder GmbH
Centrifuges	Biofuge fresco, Heraeus Instruments Biofuge pico, Heraeus Instruments Biofuge stratos, Heraeus Instruments Megafuge 1.0R, Heraeus Instruments
Cryostat	CM3050S, Leica GmbH
Digital gel documentation	ChemiDoc MP imaging systems, Bio-Rad Laboratories
Electrophoresis chamber (agarose gels)	Sub-Cell GT System, Bio-Rad Laboratories
Home cage activity measurement	Mouse-E-Motion, Infra-e-motion, Henstedt–Ulzburg,
Laminar flow hood	Herasafe, Kendro
Liquid handling platform	Janus®, Perkin Elmer
Magnetic stirrer	MR 3001 K, Heidolph, Fisher
Microplate analyzer	MRX TC II, Dynex Technologies
Microscope	Eclipse TS 1000, Nikon Axiovert 200 M fluorescent microscope, Zeiss Leica SP8 Confocal, Leica Microsystems
Open-field test device	Open-field ActiMot, TSE Systems
PCR cycler	iCycler, Bio-Rad Laboratories
pH meter	inoLab, WTW
Real-time PCR cycler	LightCycler® 480 Instrument II, Roche

Spectrophotometer	NanoDrop 1000, Thermo scientific
SpeedVac SPD111V	Thermo Fisher
Sterilizing oven	Varioklav 25T, H+P Labortechnik
Tissue homogenizer	Precellys 24, Bertin Technologies
Ultrasonic bath	Ultrasonic cleaning bath USC-THD, VWR
Vortexer	Vortex-Genie 2, Scientific Industries

2.2 Chemicals and reagents

2.2.1 Chemicals

Chemicals	Company
Albumin bovine Fraction V, pH 7.0 standard grade, lyophil. (BSA)	Serva
Brilliant Blue R-250	Sigma-Aldrich
1-Bromo-3-chlorpropane (BCP)	Sigma-Aldrich
BrdU (B5002-250mg)	Sigma-Aldrich
DAPI Fluoromount-G®	SouthernBiotech
Ethidium bromide solution (10 mg/ml)	Sigma-Aldrich
Fluoromount-G®	SouthernBiotech
2-Methylbutan/ Isopentan	Sigma-Aldrich
Paraformaldehyd	Sigma-Aldrich
Sucrose	Sigma-Aldrich
TRIzol® Reagent	Thermo Fisher
Tween20	Sigma-Aldrich
Tamoxifen	Sigma-Aldrich

2.2.2 Kits

Kits	Company
GoTaq® Green Master Mix (PCR)	Promega
BCA Protein Assay Kit	Thermo Fisher (Pierce™)
Corticosterone Enzyme Immunoassay Kit	ArborAssays

2.2.3 Buffers and solutions

If not stated otherwise all buffers and solutions were prepared with sterile dH_2O and all chemicals were purchased from Applichem, Life Technologies, Merck, Carl Roth or Sigma-Aldrich.

Buffer and solution	Composition	Application
Borate buffer	0.1 M Boric acid adjusted to pH 8.5	Immunohistochemistry
Citrate buffer	10 mM Citric acid 0.05% (v/v) Tween 20 adjusted to pH 6.0	Immunohistochemistry
Mouse tail lysis buffer	100 mM Tris/HCl pH 8.0 5 mM EDTA 200 mM NaCl 0.2% (w/v) SDS	Mouse tail lysis
2x SSC	0.3 M NaCl 30 mM Na-citrate dihydrate adjusted to pH 7.0	Immunohistochemistry
TAE buffer	40 mM Tris-acetate 1mM EDTA pH 8.0	Agarose gel electrophoresis
TBS (Tris-buffered saline)	50 mM Tris-HCl 150 mM NaCl adjusted to pH 7.5	Immunohistochemistry

TE buffer	10 mM Tris 1 mM EDTA, pH 8.0 adjusted to pH 7.4	DNA isolation
PB (Phosphate buffer)	0.02 M Sodium phosphate monobasic 0.08 M Sodium phosphate dibasic Adjusted to pH 7.4	DAGLa Immunohistchemistry
PBS (Phosphate-buffered saline)	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 Adjusted to pH 7.4	Immunohistochemistry
4% PFA	4% (w/v) Paraformaldehyde in PBS	Fixation of brain tissue
Cryoprotectant	50% PBS 25% glycerol 25% ethylene glycol 0.025% sodiumazide	Storage of free-floating sections

2.2.4 Enzymes and antibodies

Enzyme/ antibody/ serum	Company
Proteinase K	NEB
Superscript II Reverse Transcriptase	Invitrogen
Taq Polymerase	NEB
Phusion High Fidelity DNA Polymerase	NEB
Anti-DAGLa (DGLa-Rb-Af380, rabbit, polyclonal)	Frontier Institute Co.Ltd
Anti-BrdU (ab6326, rat, monoclonal, AB_305426	Abcam
Anti-CD-31 (AB_393571, mouse, polyclonal)	Abcam
Anti-GFAP (ab53554, goat, polyclonal)	Abcam
Anti-Iba1 (019-19741, rabbit, polyclonal)	Abcam
Anti-Ki67 (ab66155, rabbit, monoclonal)	Abcam
Anti-NeuN AlexaFluor® 488 (MAB377X, mouse, monoclonal, AB_2149209)	Merck Millipore
Anti-S100b (ab41548, rabbit, polyclonal)	Abcam
Donkey anti-goat AlexaFluor®488 (A-11055, AB_10564074)	Life Technologies
Donkey anti-rabbit AlexaFluor®488 (A-21206, AB_10049650)	Life Technologies
Goat anti-rabbit AlexaFluor®594 (A-11037, AB_10561549)	Life Technologies
Goat anti-rat AlexaFluor®594 (A-11007, AB_10561522)	Life Technologies
Normal goat serum (ab7481)	Abcam
Normal donkey serum (ab7475)	Abcam

2.3 Animals

In this study, conditional Dagla KO mice on a C57BL/6J genetic background (2-5 month old) were used for molecular and behavioral analysis. The Cre/loxP system was used to create cell-specific KO of *Dagla* in neurons (Syn-Dagla KO), astrocytes (GLAST-CreERT2-Dagla and GFAP-

Dagla KO) as well as in microglia cells (Cx3Cr1-CreERT2-Dagla and LyzM-Dagla KO). Therefore, mouse lines expressing Cre under specific promoters described below were crossed with *Dagla* "floxed" mice that carry two loxP sites flanking exon 1 of the *Dagla* gene (Jenniches et al. 2016a). These loxP sites allow Cre-mediated excision of the flanked exon. Cre recombinase derived from P1 bacteriophage catalyzes the recombination between the loxP sites leading to excision of the flanked gene sequence (Hoess, Abremski, and Sternberg 1984).

Mice were always bred hemi-/heterozygous for Cre to obtain mixed offspring with some expressing Cre and the others not. For all experiments Cre-negative littermates were used as controls. Hereafter, littermate controls not expressing the *Cre* gene (Dagla fl/fl and wild type for Cre) are named as "Promoter name-Dagla **WT**" serving as controls to the "Promoter name-Dagla **KO**" mice.

Mice were housed under a 12-hour light/dark cycle with *ad* libitum access to food and water (lights on at 9). All behavioral test were conducted during the dark-phase. All experiments were approved by the North Rhine-Westphalia State Environment Agency (AZ: 84-02.04.2017.A234).

2.3.1 Dagla fl/fl mice

Dagla fl/fl (B6-cg(Dagla)tm1Zim) mice were generated in our institute (Jenniches et al. 2016a). The *Dagla* gene was mutated by inserting two loxP sites flanking exon 1 to allow Cre-mediated excision of the gene fragment. Mice are backcrossed onto a C57BL/6J genetic background.

2.3.2 Neuron-specific Dagla KO mice

2.3.2.1 Syn-Dagla mice

Syn-Cre mice from The Jackson Laboratory (B6.Cg-Tg(Syn1cre)671Jxm/J) specifically express Cre recombinase (bacteriophage P1) in neuronal cells controlled by the rat Synapsin I promoter. Cre activity is detected in neuronal cells (brain, spinal cord and dorsal root ganglia) at embryonic day 12.5 the earliest. The transgene integrated into chromosome 6 over 1 Mb from the nearest gene.

Syn-Cre transgenic mice were bred with Dagla fl/fl mice (B6.Dagla tm1Zim) (Jenniches et al. 2016a), which results in a KO of DAGLa in Syn expressing cells described above. Syn-Dagla mice

could just be bred maternally (Cre-postive mother), since Cre expression was observed in the male germline.
To exclude that offspring of Syn-Dagla KO mothers develop a depressive-like phenotype due to impaired maternal care, Cre wild-type foster mothers were added to each breeding cage. Additionally, we only used littermates raised by the same mothers as controls for all behavior tests, to ensure that anxiety or depressive-like phenotypes were not affected by maternal care. To decrease the mortality of Syn-Dagla KO mothers, plastic houses were put into breeding cages.

2.3.3 Astrocyte-specific Dagla KO mice

2.3.3.1 GFAP-Dagla mice

GFAP-Cre mice from The Jackson Laboratory (B6.Cg-Tg(Gfap-Cre)73.12Mvs/J) from founder line 73.12 express Cre recombinase regulated by the mouse glial fibrillary acid protein (GFAP) promoter. Cre expression was observed by The Jackson Laboratory in astrocytes in the brain and spinal cord, as well as postnatal and adult GFAP-expressing neural stem cells and their progeny in hippocampus dentate gyrus and subventricular zone. Because of the Cre expression in all adult neural stem cells, Cre activity was also observed in hippocampal pyramidal neurons and few cerebellar granule neurons (less than 5%). Jackson Lab reports Cre expression in the male germline and suggests breeding maternally; using GFAP-Cre female and floxed male mice (https://www.jax.org/strain/012886).
In the current project, GFAP-Cre mice from Jackson were crossed with Dagla fl/fl mice (B6.Dagla tm1Zim) resulting in a KO of DAGLa in GFAP expressing cells described above. During the breeding, we found that maternal GFAP-Cre led to germline transmission, contrary to what was found by The Jackson Laboratory. Furthermore GFAP-Cre x Dagla fl/fl breeding seems to give offspring less frequently compared to other Cre-Dagla lines, fitting with the decreased motivation observed in these mice (see Results).

2.3.3.2 GLAST-CreERT2-Dagla mice

GLAST-CreERT2 (Slc1a3tm1(cre/ERT2)Mgoe) mice with a tamoxifen-inducible form of Cre (CreERT2) expressed in the locus of astrocyte-specific glutamate aspartate transporter (GLAST), were generated by Mori *et al.*, 2006 (Mori et al. 2006a). A fusion of Cre recombinase to the ligand binding domain of the modified estrogen receptor (ERT2) ensures that the complex can only translocate into the nucleus upon tamoxifen stimulation, where it can mediate

recombination. Without tamoxifen stimulation the complex will stay in the cytoplasm (Feil et al., 1996). This allows the induction of recombination at specific time points in adult astrocytes, radial glia cells and adult neural stem cells. Mori *et al.* found a recombination efficiency of around 1/3 of all astrocytes and a specificity of approximately 80%.
For the breeding of the GLAST-CreERT2-Dagla line, we used non-induced maternal or paternal breedings.

2.3.3.2.1 Tamoxifen treatment of GLAST-CreERT2-Dagla mice

To induce the *Dagla* KO in astrocytes of GLAST-CreERT2-Dagla mice, 7-9 weeks old mice were treated with the estrogen receptor antagonist tamoxifen.
Therefore, tamoxifen was dissolved in corn oil (and ethanol) at 37 °C to prepare a 20 mg/mL solution. Solutions were sonicated for better solubility for 10 min. Mice were injected twice per day with 50 µL of the tamoxifen solution (1mg/day) for five consecutive days. Injections were performed intraperitoneally to both sides alternately with at least 6 hours between the first and second injection in day. This protocol was tested as the most efficient to induce Cre-activity by Mori *et al.* 2006. Mice entered the experiment the earliest at 3 weeks after the injection to ensure full induction of the KO.

2.3.4 Microglia-specific Dagla KO mice

2.3.4.1 LyzM-Dagla mice

LyzM-Cre (B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J) mice from The Jackson Laboratory express Cre recombinase under the control of the LyzM-promoter. This promoter is active in the myeloid cell lineage, including monocytes, mature macrophages and granolocytes. The Cre line was characterized by Orthgiess *et al.,* 2016 using RosaTomato reporter line (Orthgiess et al. 2016). They found a very low LyzM-Cre mediated tdTomato expression in microglia with brain region specific effects. In hippocampus 18% and in cerebellum 27% of Iba1-positive cells were expressing tdTomato, whereas only 5% of microglia in motor cortex showed tdTomato expression. The overall efficacy of the promoter was 10.7%. They also found an off-target effect of the promoter in neurons. In the motor cortex, approximately 10% of all neurons were expressing tdTomato, whereas 20% of granule cells in the cerebellum exhibited tdTomato expression. In CA3, 10% of neurons were tdTomato positive, but none in CA1 or CA2. LyzM promoter is not active in neuronal precursor cells, but is activated in neurons when these cells are already restricted to neuronal linage.

In all peripheral organs Orthgiess *et al.* tested (spleen, adipose tissue, liver, and lung) almost all peripheral macrophages, identified by Iba1 staining, showed tdTomato co-expression.

LyzM-Dagla mice could be bred paternally or maternally without any germline transmission.

2.3.4.2 Cx3Cr1-CreERT2-Dagla mice

Cx3Cr1-CreERT2-Cre (B6.129P2(C)-Cx3Cr1$^{tm2.1(cre/ERT2)Jung}$/J) mice from The Jackson Laboratory express a tamoxifen-inducible form of the Cre protein (CreERT2), under the endogenous Cx3Cr1 (chemokine (C-X3-C motif) receptor 1) promoter in the mononuclear phagocyte system, as well as in microglia. The targeting vector was designed to replace exon 1 of the Cx3Cr1 gene with a Cre-recombinase fused to a mutated form of the estrogen receptor ligand binding domain. The Cre line was validated using Rosa-Tomato reporter mice (Yona et al. 2013). In those mice, Cre-mediated recombination resulted in fluorescent protein expression in peripheral myeloid cells, macrophages and microglia. Additionally, they found low levels of Cre activity prior to tamoxifen treatment, but Cre activity was significantly greater after tamoxifen treatment.

For our experiments Cx3Cr1-CreERT2 mice were bred into the Dagla fl/fl background to produce a *Dagla* KO in the phagocyte system and in microglia cells. Non-induced mice were bred paternally or maternally.

2.3.4.2.1 Tamoxifen treatment of Cx3Cr1-CreERT2-Dagla mice

To induce the *Dagla* KO in Cx3Cr1-CreERT2-Dagla mice, 7-9 weeks old mice were treated with tamoxifen. Therefore, tamoxifen was prepared as described in section 2.3.3.2.1. Mice were injected subcutaneously under isoflurane anesthesia in the area of hips and shoulders (50 µL tamoxifen solution per injection (200 µL) = 4 mg/day). Injections were repeated after 48 hours. After injections, mice were kept for at least 4 weeks before they were tested in any experiments to induce the KO and ensure that peripheral myeloid cells with the deletion are renewed to express *Dagla* (Goldmann et al. 2013).

2.3.5 Rosa-Tomato reporter line

To validate which cell types express Cre in our mouse lines, the RosaTomato reporter line from The Jackson Laboratory (B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J) was used. The reporter construct was inserted into a Gt(ROSA)26Sor locus by homologous recombination

(https://www.jax.org/strain/007914). RosaTomato mice exhibit a loxP-flanked STOP cassette preventing transcription of CAG promotor-driven red fluorescent protein tdTomato. TdTomato is expressed when bred to mice that express Cre recombinase. For validation of the lines, we used mice that were heterozygous for RosaTomato (wt/ins) and Cre (wt/tg).

2.4 Molecular biology

2.4.1 Mouse genotyping

2.4.1.1 DNA isolation

For genotyping mice were earmarked directly after weaning at an age of 3 weeks. At the same time a small piece of the tail was cut off and transferred into a reaction tube. Tail tips were incubated with 200 µL lysis buffer and 1 mg/mL of proteinase K overnight at 55 °C with shaking (550 rpm) for cell lysis. To remove cell debris, samples were centrifuged at 13000 rpm for 10 min and the supernatant was transferred into a fresh tube. By adding equal volume of isopropanol to each sample, DNA was precipitated. Samples were inverted for better distribution of isopropanol and subsequently centrifuged at 13000 rpm for 10 min. The DNA pellet was washed two times in 70% EtOH followed by centrifugation. Lastly, DNA pellets were dried and then diluted in 40-100 µL TE-buffer, depending on achieved pellet size. Isolated DNA was stored at 4 °C.

The concentration of DNA was determined using a photospectrometer (NanoDrop1000, Thermofisher). DNA absorbs UV light at a wavelength of 260 nm. The absorbance of 1 unit at 260 nm is equivalent to a DNA concentration of 50 µg/mL. The purity of DNA was assessed by the ratio of absorbance at 260 and 280 nm. The ratio for very pure DNA is approximately 1.8.

2.4.1.2 DNA amplification by Polymerase Chain Reaction (PCR)

The amplification of specific DNA fragments for genotyping was executed with the polymerase chain reaction (PCR).

During one PCR cycle DNA is heated to 94-96 °C to denaturate double-stranded DNA. Once the stands are separated, the sample is adjusted to a temperature that is appropriate for primer annealing. This temperature is depending on the lengths and the GC content of the primers used. After the attachment of primers, a heat stable polymerase (Taq-Polymerase) is generating the complementary DNA strand starting at the primer sequence. The temperature during this

elongation step is adjusted to the optimal working temperature of the polymerase (68–72°C). With each number of cycles, DNA products are amplified exponentially.
For all genotyping reactions, 10 x ThermoPol reaction buffer (BioLabs) was used. Established PCR conditions and primers are listed in the following paragraphs.

2.4.1.2.1 Cre-Recombinase PCR

To test the presence of a Cre-recombinase gene, two Cre PCRs were performed in parallel. Primer pairs of Cre1 and Cre 2 PCR are binding to different locations in the Cre gene, to ensure the reliability of PCR results. GAPDH primers were used as positive control, while water was used instead of DNA as negative control. The following reagents, primers and conditions were used:

MilliQ water	14.3 µL
10 x ThermoPol buffer	2 µL
dNTPs	0.5 µL
Cre1_fwd Primer (5´-CATTTGGGCCAGCTAAACAT -3´)	0.5 µL
or	
Cre2_fwd Primer (5´-GCATTTCTGGGGATTGCTTA -3´)	0.5 µL
Cre1_rev Primer (5´- CCCGGCAAAACAGGTAGTTA-3´)	0.5 µL
or	
Cre2_rev Primer (5´ -TGCATGATCTCCGGTATTGA -3')	0.5 µL
GAPDH_fwd Primer (5´-CCACTGAAGGGCATCTTGGG -3´)	0.5 µL
GAPDH_rev Primer (5´-CAGCTGGATGTCAGAGCCAATAA-3´)	0.5 µL
DNA (100-150 ng/µL)	1 µL
Taq- Polymerase	0.2 µL

PCR program:

1 x	Initial denaturation	94°C	3 min
30 x	Denaturation	94°C	30 s
	Annealing	60°C (Cre 1)/ 57°C (Cre 2)	60 s
	Elongation	68°C	60 s
1 x	Final elongation	68°C	7 min
1 x	Cooling	4°C	∞

The presence of Cre gene led to a 454 bp fragment in Cre1- PCR and a 449 bp fragment in Cre2-PCR. GAPDH control DNA fragment has a size of 604 bp and should be present in each sample.

2.4.1.2.2 DAGLa-KO PCR

To detect, if the *Dagla* gene was floxed, wild-type or deleted, Dagla-KO PCR was performed. For all conditional KO lines, *Dagla* gene needs to be floxed. The following conditions and primers were used for this PCR:

MilliQ water	14.3 µL
10 x ThermoPol buffer	2.5 µL
dNTPs	0.5 µL
KO_fwd Primer (5´-TAGCTTAGCCCCCATGTGAC-3´)	0.5 µL
KO_rev Primer (5´-CCCAGTAGCCACAGAACCAT-3´)	0.5 µL
WT_fwd Primer (5´-GAGATGGGTCCACCTCCTT-3´)	0.5 µL
DNA (100-150 ng/µL)	1 µL
Taq- Polymerase	0.2 µL

PCR program:

1 x	Initial denaturation	95°C	30 s
30 x	Denaturation	94°C	30 s
	Annealing	56°C	60 s
	Elongation	68°C	60 s
1 x	Final elongation	68°C	5 min
1 x	Cooling	°C	∞

If the *Dagla* gene contained a loxP site, the PCR product had a size of 450 bp, the wild-type allele 403 bp and if the *Dagla* gene was deleted, 204 bp.

2.4.1.2.3 Promoter-specific Cre PCRs

With the standard Cre PCR it is possible to show, if the Cre recombinase gene is present or not. To show, whether Cre is expressed under the control of a certain promoter, promoter-Cre PCRs were used. The following table shows the conditions and the primers used for different promoter-specific Cre PCRs.

Syn-Cre				
Mix:		**PCR-program**		
MilliQ water	13.6 µL	1x	95°C	02:00
10 x ThermoPol buffer	2 µL	30x	95°C	00:15
			64°C	00:15
			68°C	00:20

dNTPs	0.4 µL	1x	68°C	01:00
MgCl2	0.8 µL	1x	4°C	∞
hSyn Primer (5´-CTCAGCGCTGCCTCAGTCT-3´)	1 µL			
Cre Primer (5´-GCATCGACCGGTAATGCA-3´)	1 µL			
DNA (100-150 ng/µL)	1 µL			
Taq- Polymerase	0.2 µL			

Presence of Syn-Cre gene resulted in a PCR product of 300 bp.

GFAP-Cre				
Mix:		**PCR-program**		
MilliQ water	13.6 µL	1x	95°C	02:00
10 x ThermoPol buffer	2 µL	30x	95°C	01:00
			60°C	02:00
			68°C	01:00
dNTPs	0.4 µL	1x	68°C	05:00
MgCl2	0.8 µL	1x	4°C	∞
GFAP-Cre_fwd Primer (5´-ACTCCTTCATAAAGCCCTCG-3´)	1 µL			
GFAP-Cre_rev Primer (5´-ATCACTCGTTGCATCGACCG-3´)	1 µL			
DNA (100-150 ng/µL)	1 µL			
Taq- Polymerase	0.2 µL			

The presence of GFAP-Cre gene resulted in a PCR product of 190 bp.

LyzM-Cre				
Mix:		**PCR-program (Touchdown)**		
MilliQ water	14.6 µL	1x	94°C	02:00
10 x ThermoPol buffer	2 µL	10x	94°C	00:30
			65°C (-0.5 C per cycle)	01:00
			68°C	01:00
dNTPs	0.4 µL	28x	94°C	00:30
			60°C	01:00
			68°C	01:00
MgCl2	0.8 µL	1x	68°C	02:00
oIMR3066 Primer (5´-CCCAGAAATGCCAGATTACG-3´)	0.5 µL	1x	4°C	∞
oIMR3067 Primer (5´-CTTGGGCTGCCAGAATTTCTC-3´)	0.5 µL			
DNA (100-150 ng/µL)	1 µL			
Taq- Polymerase	0.2 µL			

The presence of LysM-Cre gene resulted in a PCR product of 700 bp.
Primers for Cx3Cr1 and GLAST-promoters bound unspecifically to Cre under different promoters and thus could not be used for validation of these lines.

2.4.1.3 Agarose gel electrophoresis

To separate and identify PCR products by size, an agarose gel electrophoresis was conducted. 1.5% agarose was dissolved in TAE buffer by heating in the microwave. After dissolving, the solution was poured into a gel chamber with a comb inserted to form wells. When the gel was hardened, it was located into an electrophoresis chamber filled with TAE buffer. 20 µL of the samples mixed with 2 µL of loading dye and 5 µL of 100 bp ladder were loaded in the wells on the gel. Electrophoresis was executed for 45 min at 120 V. DNA migrates through the pores of the gel into the direction of the positive pole due to its negative charge. Smaller DNA fragments migrate faster through the gel than the bigger fragments resulting in the separation by size.
After electrophoresis, agarose gels were placed in an ethidium bromide bath (1.5 µg/mL) for 20 min. EtBr intercalates into the DNA fragments in the gel and was detected using the ChemiDoc MP imaging systems at 300 nm.

2.4.2 mRNA expression analysis by TaqMan

2.4.2.1 RNA purification and measurement

Total RNA from frozen brain or liver tissue was extracted using Trizol® Reagent (Life Technologies). Tissue was transferred into 2 mL tubes containing ceramic beads (Peqlab). Per 100 mg of tissue 1 mL of Trizol® was added and samples were homogenized by shaking in a homogenizer. After short centrifugation the homogenate was transferred into a fresh tube without the ceramic beads. To remove cell debris, the solution was centrifuged at 12000 *g* for 10 min at 4˚C. The supernatant was transferred into a new tube and 1:10 volume of 1-bromo-3-chloropropane (BCP) was added to separate RNA and DNA from proteins and lipids. After vortexing for 30 s and incubation for 3 min at RT, samples were centrifuged (12000 *g*, 10 min, 4˚C) and the RNA-containing upper phase was collected in fresh tubes. For RNA precipitation isopropanol was added (1:1) and the samples were centrifuged again after vortexing. The RNA pellet was washed two times in 1 mL 75% EtOH before RNA was dried at RT for 30 min. Depending on pellet size RNA was dissolved in 20-50 µL RNase-free water.
Total RNA concentration and purity were determined using a spectrophotometer (NanoDrop). The spectrophotometer is measuring the optical density of the sample when exposed to ultraviolet light at a wavelength of 260 nm to determine the concentration. To evaluate the purity of RNA samples, the ratio of absorbance at 260/280 nm was measured. For pure RNA the ratio is approximately 2. RNA was stored at -80˚C.

2.4.2.2 DNA digestion

To exclude binding of TaqMan primers to genomic DNA, the possible remnants of DNA in the samples were digested using DNaseI treatment. For treatment of 1 µg of RNA, 1 U of DNaseI and 10x incubation buffer were added and incubated for 30 min at 26˚C. For enzyme inactivation the samples were incubated at 75˚C for 5 min.

2.4.2.3 Reverse transcription polymerase chain reaction

For reverse transcription of isolated RNA, all RNA samples were diluted to a final volume of 11 µL with each sample containing the same concentration of RNA. Subsequently, 1 µL of Oligo(dt)$_{12-18}$ primer (Life Technologies) and 1 µL of Desoxynucleotide Mix (10mM, Sigma-Aldrich) were added to each reaction tube and incubated for 5 min at 65˚C for primer annealing. After cooling

down, samples were incubated at 4˚C for at least 3 min. 4 µL of 5x first strand buffer (Life Technologies) and 2 µL of 0,1 M DTT (Life Technologies) were added. DTT serves as a stabilizer of reverse transcriptase. Samples were incubated at 42˚C for 2 min and then cooled down to 4˚C for at least 3 min. Subsequently, 1 µL reverse transcriptase was added to each reaction tube. The following cycling parameters were used for reverse transcription of RNA into cDNA:

42˚C	50 min	Enzyme reaction
70˚C	15 min	Enzyme inactivation
4˚C	10 min	Cooling

Produced cDNA was adjusted to a final concentration of 20 ng/µL and stored at -20˚C.

2.4.2.4 Real-time reverse transcription PCR (qRT-PCR)

Differences in mRNA levels were determined using TaqMan® Gene expression Assays (Applied Biosystems). TaqMan probes are gene-specific oligonucleotides, covalently coupled with a fluorescent molecule (FAM (6-carboxyfluorescein)) at the 5´end and a quencher molecule at the 3´end. The quencher molecule quenches the fluorescence emitted by the FAM molecule when exposed to the cycler´s light source via Förster resonance energy transfer (FRET) and inhibits all fluorescence signals. As soon as the gene is amplified, the exonuclease activity of Taq-Polymerase cleaves the probe from the complementary target sequence and separates the fluorescent molecule from the quencher. With each PCR cycle more fluorescent molecules are released which leads to an increase of fluorescence signal proportional to the amount of cDNA.

TaqMan® analysis was conducted in 384 well-plate with triplicates of each sample. A total amount of 80 ng of cDNA was used per well and 6 µL of the master mix containing 5 µL TaqMan® Gene expression Master Mix (Applied Bioscience), 0.5 µL 20xTaqMan® Gene Expression Assay Mix (Primer) (Table 1) and 0.5 µL of RNA free water. The plate was placed into the LightCycler®480 (Roche) and cDNA was amplified via Real-Time PCR with 45 cycles (program: initial denaturation: 95°C for 10 min; 45 cycles of 95°C for 15 s and 60°C for 1 min; cooling to 4°C).
Hypoxanthin-phosphoribosyl-transferase (*Hprt*) was used as the housekeeping gene and water as control. Expression levels were calculated with the advance relative quantification (2^{-ddCt}) setting in the software.

Table 1: Gene Expression Assay mix list. Provided by ThermoFisher Scientific

Target mRNA	Gene name	TaqMan Assay ID
Abhd12	Abhydrolase domain containing 12	Mm00470489_m1
Abhd6	Abhydrolase domain containing 6	Mm00481199-m1
Avp	Vasopressin	Mm00437761_g1
Bdnf	Brain derived neurotrophic factor	Mm01334042_m1
Cnr1	Cannabinoid receptor 1	Mm01212171_s1
Cnr2	Cannabinoid receptor 2	Mm00438286_m1
Crh	Corticotropin releasing hormone	Mm01293920_s1
Crhr1	Corticotropin releasing hormone receptor 1	Mm00432670_m1
Dagla	Diacylglycerol lipase alpha	Mm00813830_m1
Daglb	Diacylglycerol lipase, beta	Mm00523381_m1
Faah	Fatty acid amide hydrolase	Mm00515684_m1
Fgf2	Fibroblast growth factor 2	Mm01285715_m1
Nr3c1	Glucocorticoid receptor	Mm00433832_m1
Hprt VIC	Hypoxanthine guanine phosphoribosyl transferase	Mm01545399_m1
Igf2	Insulin-like growth factor 2	Mm00439564_m1
Il-10	Interleukin 10	Mm00439614_m1
Il-1ß	Interleukin 1 beta	Mm00434228_m1
Il-6	Interleukin 6	Mm00446190_m1
Magl	Monoglyceride lipase	Mm00449274_m1
Oxt	Oxytocin	Mm01329577_g1
OxtR	Oxytocin receptor	Mm01182684_m1
Tnfa	Tumor necrosis factor	Mm00443258_m1
Vegf	Vascular and endothelial growth factor	Mm00437306_m1

2.5 RNAscope assay

RNAscope *in situ* hybridisation to validate the efficacy of *Dagla* deletion in the cells of interest (www.acdbio.com/RNAScope) was performed in collaboration with Dr. Este Leidmaa. For this

purpose, mice (n = 3-4 per genotype) were killed by decapitation, brains were quickly removed, flash frozen in dry ice-cooled isopentane and stored in -80 °C. Brains were cryosectioned at a thickness of 10 µm and mounted on SuperFrost Plus slides (Thermo Fisher). The exact Bregma coordinates were identified from every twelfth slide according to Paxinos Brain atlas (Franklin and Paxinos 2008). RNAscope Multiplex Fluorescent Reagent kit was used according to manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA). The probes for *Dagla* mRNA (cat.no 478821) were multiplexed with the probes detecting a specific pan-astrocytic marker Aldh1l1 (cat.no 405891-C2) (Srinivasan et al. 2016) and a well-established neuronal marker Rbfox3 (cat.no 313311-C3). Confocal images of sections were obtained using a Leica TSC SP8 with 40x magnification (291 µm^2 area) and analyzed using Fiji software (version 2.0.0-rc-69; ImageJ). Cell nuclei were manually encircled and selected as regions of interest from an image with DAPI, Aldh1l1 and Rbfox3 signals. Nuclei with at least 2 dots of Aldh1l1 and no Rbfox3 signal were designated as astrocytes and nuclei with at least 2 Rbfox3 and no Aldh1l1 particles as neurons. The same number of astrocytes and neurons were counted on a picture (n=24-30 cells per animal, depending on the brain region). The numbers of Dagla particles per selection (astrocytes or neurons) were then quantified. Micrographs from at least two consecutive sections from an animal were analyzed per brain region. Sections from constitutive Dagla KO mice (Jenniches et al. 2016a) were used as controls for non-specific binding of the Dagla probe.

2.6 Immunohistochemistry

2.6.1 DAGLa staining

To detect DAGLa protein in the brain and confirm the KO-efficacy in our mouse-lines, a DAGLa immunohistostaining was performed. Therefore, mice were anesthetized with isoflurane before they were transcardially perfused with 20 mL of cooled PBS to remove blood from the body, followed by 60 mL of 4% formaldehyde to fix proteins. The brains were post-fixed in 4% formaldehyde for 24 hours, incubated in 20% sucrose/PBS for approximately 3 days, and frozen in dry ice-cooled isopentane. Tissues were stored at -80 °C until further processing.

Brains were cut into 40 µm thick free-floating sections (Bregma -0.94 to -3.34 mm) in the cryostat with a chamber temperature of -22 °C and an object plate temperature of -21 °C. The brain slices were collected in 48 well plates filled with cryoprotectant and stored at -20 °C until they were stained.

For immunofluorescence staining, 0.2M phosphate buffer (PB, pH 7.6) was used as a diluent and wash solution inbetween the following steps of the staining procedure. 0.3% Triton was used for permeabilization (10 min). Non-specific antibody binding was blocked with a solution containing 5% normal goat serum (NGS) and 0.2% Triton (1 hour at RT), followed by the staining with anti-DAGLa antibody (DGLa-Rb-Af380; Frontier Institute Co., Ltd., Hokkaido, Japan) overnight at RT (1:400 in blocking solution). Secondary goat anti-rabbit Alexa-Fluor647 was incubated for 2 h at RT (1:500 in blocking solution, Life Technologies). Stained brain slices were quickly washed in ddH20 before they were embedded in DAPI Fluoromount-G(R) media (Southern Biotechnology Associates. Inc.). Fluorescent images were obtained at the Leica SP8 confocal microscope with 20x or 40x objective lenses.

2.6.2 RosaTomato Co-staining

To validate the different Cre-lines and to identify in which cell type Cre is expressed, RosaTomato reporter mice were used (described in 2.3.5). The RosaTomato reporter line expresses a red fluorescent protein tdTomato in those cells where Cre recombinase is active. To identify the cell type that is expressing tdTomato, immunohistochemical stainings with different cell markers (GFAP, S100b, NeuN, Iba1) were performed.

RosaTomato$^{wt/ins}$Promotor-Cre$^{wt/tg}$ mice were perfused transcardially with PBS followed by 4% PFA. After perfusion brains were isolated and postfixed in 4% PFA for one hour before they were cryoprotected in 20% sucrose overnight at 4 °C. Brains were frozen in dry-ice cooled isopentane and stored at -80 °C. Brains were cut into 16 µm slices and collected on object slides for staining. Before the staining, object slides were dried on a heating plate at 38 °C for 30 min. Brain slices were washed 3 times for 5 min in PBS and were permeabilized by 0.5% TritonX100 in PBS under stirring for 1 h. Another washing step followed. To prevent unspecific binding, antigens were blocked in 3% BSA for 2 hours prior first antibody incubation. Prior to primary antibody incubation slices were washed three times for 10 min. All antibodies were diluted in 0.3% BSA/PBS. To label microglia, rabbit anti-Iba1 antibody (WAKO, 1:2000) was used. To label astrocytes rabbit-anti-S100b (Abcam, 1:2000) and chicken anti-GFAP (Abcam, 1:2000) antibodies were used and to identify neurons rabbit anti-NeuN antibody coupled to Alexa-Fluor 488 (Merck, Millipore, 1:500) was used. First antibody incubation was conducted for 24 hours at 4°C. Afterwards slices were washed three times for 10 min and shortly blocked again in 3% BSA. To detect the primary antibodies, anti-rabbit Alexa-Fluor 488 (Life Technologies, 1:1000), donkey anti-rabbit Alexa-Fluor 647 (Life Technologies, 1:2000) or goat anti-chicken AF488 (Life Technologies, 1:2000) were used in different stainings. Secondary antibodies were incubated for 2 hours at RT in the dark. Subsequently slices were washed three times for 10 min in PBS,

followed by a brief washing step in MilliQ water, before they were embedded in DAPI containing mounting medium (SouthernBiotech) and covered with a coverslip. Images were obtained at the confocal microscope (LeicaSP8) with 40 x magnification.
Promoter specificity was defined as percentage of tdTomato expressing cells that are also positive for the desired cell type marker. Accordingly, promoter efficacy describes the percentage of cell type marker positive cells that are co-expressing tdTomato.

2.6.3 Adult hippocampal neurogenesis

To label proliferating cells, two months old mice were injected intraperitoneally with 5-bromo-2´-deoxyuridine (BrdU; 50 mg/kg bodyweight, dissolved in sterile saline) once a day for three consecutive days. The thymidine analogue BrdU is incorporated in the DNA of dividing cells and can be detected immunohistochemically in their progeny by specific BrdU antibodies (del Rio and Soriano 1989).
Either 24 h or 21 days after the last BrdU injection, 4 mice of each group were anesthetized with isoflurane before they were transcardially perfused with cooled PBS following 4% PFA. After perfusion brains were postfixed in 4% PFA for 24 hours at 4 °C. To protect brains from freezing damages, brains were cryoprotected in 20% sucrose for three days at 4 °C before they were frozen in dry ice cooled isopentane and stored at -80°C. Subsequently the entire hippocampus (Bregma -0.94 to -3.34 mm) was cut into 48 serial coronal free-floating cryosections of 40 µM thickness. Slices were stored in 48 well plates filled with cryoprotectant (50% PBS, 25% glycerol, 25% ethylene glycol, 0.025% sodiumazide) at -20°C.
For immunohistochemical staining, every eighth hippocampal slice of each mouse was selected and transferred in one well of a 24-well-plate containing Netwell™ inserts (Costar). All following steps of the staining were done at room temperature under shaking, if not specified otherwise. First, slices were washed 3 times in TBS for 5 min to remove remaining cryoprotectant, followed by incubation in permeabilization buffer (TBS containing 0.3% Tween) for 10 minutes to dissolve membrane lipids and make cells permeable for antibodies. After another washing step (3 times TBS for 5 minutes), slices were incubated in 2x SSC for 20 minutes at 65°C for antigen retrieval and then shortly washed in ddH_2O. Since BrdU is located in the DNA, slices were incubated in 2M HCl for 30 min at 37°C to make DNA accessible for the antibody due to denaturation. To neutralize pH, slices were incubated in borate buffer for 10 min before they were washed in TBS again. Subsequently, slices were blocked in TBS plus II (TBS containing 0.3% Triton X-100, 5% goat serum, 2% BSA) for 1 h.

To investigate the proliferation of progenitor cells, brain slices of mice that were sacrificed 24 h after the last BrdU injection (D1), were incubated with anti-BrdU antibody (1:500 in TBS plus II) overnight at 4 °C under shaking.

To investigate the survival and differentiation of progenitor cells, brain slices of mice that were sacrificed 21 days after the last BrdU injections (D21) were incubated in TBS plus II solution containing anti-BrdU antibody (rat, 1:500); NeuN antibody to label neurons (mouse, conjugated with AF488; 1:500) and GFAP antibody to label astrocytes (rabbit, 1:1000). Since GFAP is also expressed in young neurons, the D21 staining was always repeated twice for each mouse line. In the second staining S100b antibody (1:2000) was used instead of the GFAP antibody. In both cases, brain slices were incubated in the antibody solutions over night at 4 °C under shaking.

On the next day, slices were washed 3 times in TBS for 5 minutes to remove remaining antibody solution. Afterwards the slices were blocked in TBS plus II for 1 hour. As secondary antibody, goat-anti-rat AF594 (1:500 in TBS plus II) was used to label BrdU antibodies and goat-anti-rabbit AF647 (1:1000 in TBS plus II) to label GFAP- or S100b-antibodies. Slices were incubated in the secondary antibody solution for 2 h at RT. After another washing step (3 times in TBS for 5 min) slices were transferred to coverslips and embedded in DAPI Fluoromount-G® (Southern Biotechnology Associates, Inc.). All blocking and antibody solutions were reused up to 3 times. Images of the dentate gyrus region were obtained with Leica SP8 Confocal microscope with 40x objective in tile-scan mode.

To estimate all BrdU-positive cells in the entire hippocampus, the following calculation was used:

$$N = \Sigma(BrdU\ cells\ per\ animal) \times \left(\frac{1}{ssf}\right) \times \left(\frac{1}{asf}\right) \times \left(\frac{1}{tsf}\right) \times 2$$

The selected sampling fraction (ssf) is 0.125 (6/48) because 6 out of 48 hippocampal slices were analyzed. Since the entire dentate gyrus was used as counting frame, the value for the area sampling fraction (asf) equals 1. Due to the thickness of slices (40 µm), the optical section of confocal microscope (1.3 µm) and an average diameter of nuclei of ~8 µm, the thickness of sampling fraction (tsf) was considered as 0.25 ((1.3 x 8)/40). Last, the calculated number of BrdU-positive cells was multiplied with two, because only one hemisphere was imaged for analysis.

Since the staining for survival and differentiation (D21) was always performed two times (with GFAP or with S100b) on 6 slices, the average amount of BrdU-positive and NeuN-positive cells per mouse of both stainings was used for analysis. Differentiation was evaluated by determination of overlaps of BrdU signal with either neuronal- (NeuN) or astrocytic (GFAP/S100b) markers.

2.6.4 Astrogliosis

Astrogliosis is an abnormal increase in the number or change in morphology of astrocytes which can occur due to inflammation, ischemia, stroke, CNS injury and neurodegenerative disease. During astrogliosis, the molecular expression and morphology of astrocytes can change, which can lead to scar formation. Scar formation is important for example to reduce the spread of neuronal inflammation or to repair the blood brain barrier, but it can also cause an inhibition of axon regeneration.

To investigate astrogliosis in our conditional KO mice, the same brain slices (stained with GFAP and DAPI) as for adult neurogenesis D21 experiments were used. The staining was performed like described in the previous paragraph 2.5.2. Per group, 4 animals were used (4wt/4tg) and 6 brain slices were analyzed per animal. The analysis was done with Fiji. First, the brightness of GFAP staining was adjusted for each picture to the same value. Afterwards an appropriate threshold was adjusted for the GFAP channel to receive a binary picture. By using the DAPI channel, masks were created to select the different regions of the dentate gyrus (see Figure 2.1): polymorph layer, granule cell layer and molecular layer.

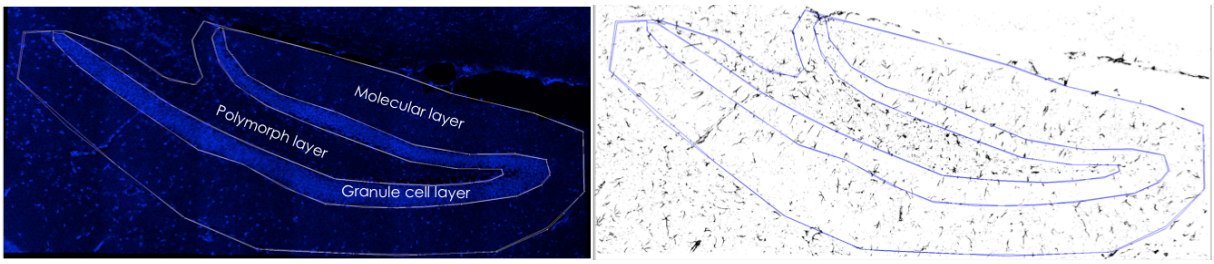


Figure 2.1: Analysis of Astrogliosis in the dentate gyrus of conditional Dagla KO mice. *In the first pictures, different areas of dentate gyrus are shown. The second picture shows a binary picture of a GFAP staining for astrocytes that was analyzed in the different regions of dentate gyrus.*

The masks were applied on the thresholded GFAP images. The area fraction, which is the percentage of area covered with GFAP, was measured.

To investigate the number of astrocytes in the different areas of the dentate gyrus, the S100b staining described in 2.5.2. was used. S100b staining brightness was adjusted and background was subtracted. An appropriate threshold was adjusted for S100b and holes were filled. By using the ´watershred´ function of Fiji, connected cells were separated from each other. Particles were counted by Fiji after application of the masks for different regions of dentate gyrus, which were again prepared using the DAPI channel. One particle was defined as dot with a size of at least 10 microns.

2.6.5 Angiogenesis

In order to investigate neurovascular coupling or general brain blood supply in conditional Dagla KO mice, an immunohistochemical staining against a blood vessel marker CD31 was performed. Therefore, mice were killed by decapitation and brains were directly frozen in isopentane. Fresh frozen brains were stored in -80 °C until they were cut in 10 µm thick slices. Series of six hippocampal slices 200 µm apart from each other were collected on one object slide which were stored in -80 °C. All following steps of the staining were done at room temperature under shaking, if not specified otherwise. Brain slices were first post-fixed in 4% PFA for 10 min. After three washing steps in TBS for 5 min, brain slices were incubated in permeabilization buffer (0.3% TritonX100 in TBS) for 10 min. Brain slices were washed three times in TBS for 5 min. Afterwards they were incubated in blocking solution (1% BSA; 0.1% TritonX100 in TBS) for one hour. The anti-CD31 (mouse, ab_393571, diluted 1:300 in blocking solution) was incubated for 24 hours at 4°C. After primary antibody incubation, brain slices were washed three times for 5 min in TBS. Secondary antibody (donkey anti-mouse AF647, 1:100 in blocking solution) was incubated for 2 hours at RT. Another washing step was performed before brain slices were covered with DAPI Fluoromount-G® (Southern Biotechnology Associates, Inc.). Images of the dentate gyrus region were obtained with Leica SP8 Confocal microscope with 20x objective in tile-scan mode. For analysis, an appropriate threshold was adjusted for CD31 channel to receive a binary picture. Using the DAPI channel, masks were created to select the dentate gyrus. The masks were applied on the respective CD31 thresholded image. The area fraction, which is the percentage of area covered with blood vessels (CD31), was measured.

2.7 Behavioral experiments

Behavioral experiments were started when mice reached an age of 2 month (experimental plan in Figure 2.2). For each mouse line (GFAP-, LyzM-, Syn-, GLAST-, and Cx3Cr1-DAGLa) both genders were tested in all behavioral tests. For inducible DAGLa-KO lines, a group size of 14 animals was used, due to a possible variability in tamoxifen-induction. For all other lines a group size of 10 animals was reasonable.

Before testing, mice were single-housed and habituated to the reverse dark/light cycle (12 hours light from 9 pm to 9 am) for at least seven days. Body weight of all experimental mice was measured during single-housing and weekly after the tests. Inducible KO lines (GLAST- and Cx3Cr1-CreERT2-DAGLa) were injected with tamoxifen either three weeks (GLAST) or one month

(Cx3Cr1) before single housing. The experiments were performed starting from the least stressful for mice and ending with the most stressful experiment. Between the experiments, mice had one week to recover from the stress of the previous test to ensure that the behavior is not influenced by stress of previous tests. All experiments were approved by the North Rhine-Westphalia State Environment Agency (AZ: 84-02.04.2017.A234).

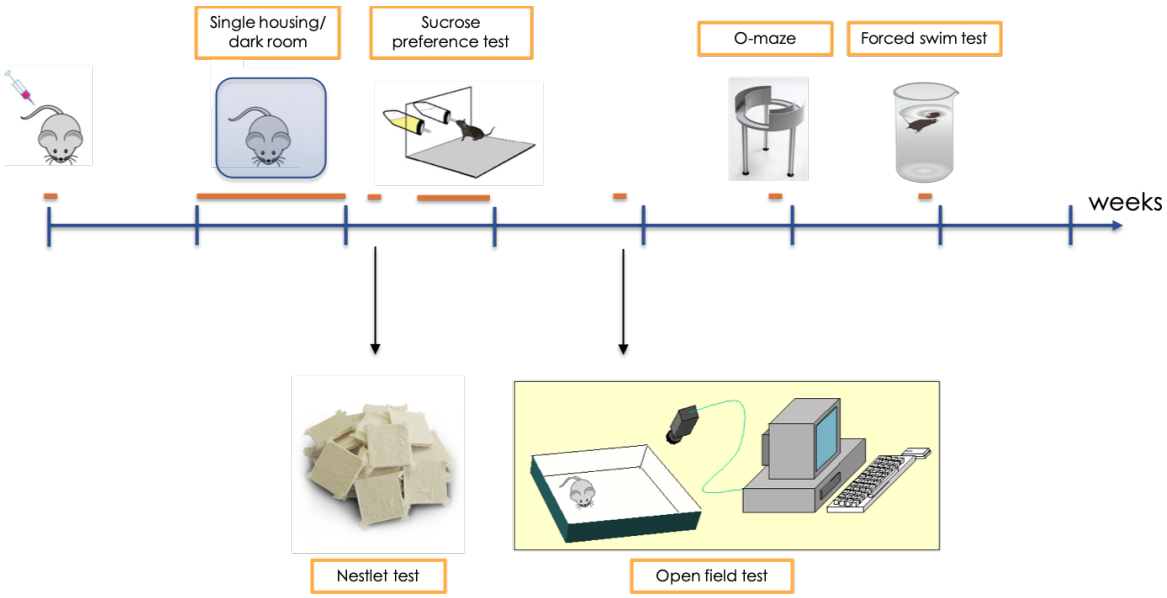


Figure 2.2: Time schedule of behavioral testing.

Inducible lines were treated with tamoxifen either 3 weeks (GLAST-CreERT2-Dagla) or 4 weeks (Cx3CR1-CreERT2-Dagla) before single-housing in the dark room. After 1 week of habituation to single-housing and the converted dark-light-cycle, the nestlet test was performed. Since mice were not interrupted in their daily routine or stressed due to the nestlet test, sucrose preference test was performed already 3 days after it. Open-field, zero-maze and forced swim tests had one week in between to let mice recover from the experiment. The behavioral tests were organized starting from the test with the mildest stress to ending with the most stressful test.

Additionally to the behavior tests shown in Figure 2.2, pup retrieval tests were performed to investigate maternal care. Therefore, maternal breedings (with tamoxifen-injected females in case of Cx3Cr1-CreERT2- and GLAST-CreERT2-Dagla KO) were used. Home-cage activity measurements were added after habituation to single-housing for GFAP-Dagla and Syn-Dagla lines, to test for hyperactivity. Moreover, Syn-Dagla KO mice were tested in light/dark test and social avoidance test, because the results of open-field test and zero-maze were not clear for this line. After the behavioral tests, mice were killed and tissue was isolated for targeted lipidomics and molecular analysis.

2.7.1 Pup retrieval test

Maternal behavior was assessed in the pup retrieval test. This test is based on the assumption that mice with depressive-like behavior might neglect their pups.
Mice were habituated to the experimental room for 30 minutes in their home cages. For the test, the entire litter as well as the male were transferred to a new cage while the Cre-positive mother was kept in the home cage. After 5 minutes, 3 pups (PN 2-6) were returned to the home-cage away from the nest at the opposite end of the cage. The latency (in seconds) to sniff the first pup and retrieve all 3 pups to the nest was measured. If the female did not retrieve all pups within 5 minutes, the test was terminated resulting in a latency of 300 seconds. The entire test was recorded on video (Handycam). To ensure that the tested females had almost the same experience in maternal care, pup retrieval test was performed with the second litter at the earliest.

2.7.2 Nestlet test

Building nests is an important behavior for mice to hide from predators, for reproduction and heat conservation. The small size of mice makes them very vulnerable to heat loss, so both males and females build nests of similar sizes (Lisk, Pretlow, and Friedman 1969). Nest building performance observed in the home cage has been proven to be valuable to assess stress, brain malfunction or damage as well as neurodegenerative disease (Gaskill et al. 2013) and is used as a parameter in models of psychiatric disorders. Nesting behavior is reduced by pain or stress. Observations in the home-cage are especially advantageous as they produce minimal stress on the animal and a good performance in these indicates a normal behavioral function and well-being in mice (Arras et al. 2007).
For the test, the old nestlet was removed and a new nestlet was weighted and placed into the home cage. After 4 and 60 hours, loose parts of the nestlet were removed and the weight of the remaining nestlet was determined. The percentage of nestlet shredded was calculated for analysis. Additionally, nests were scored according to Deacon *et al.* 2006 (Figure 2.3) (Deacon 2006).

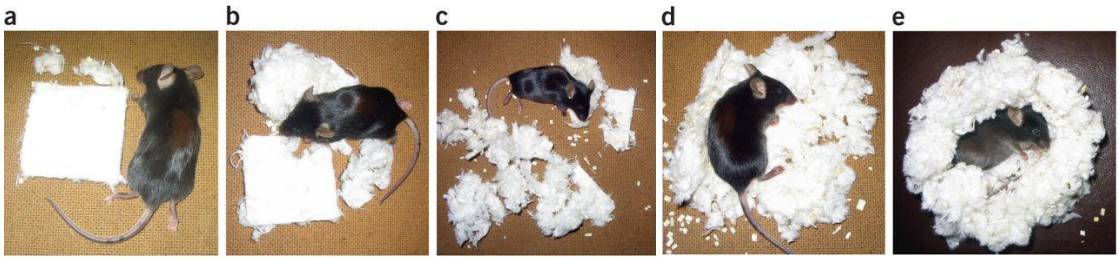


Figure 2.3: Scoring of nests according to Decan et al., 2006.

*(**a**) Mouse did not touch the nestlet resulting in a score of 1. (**b**) Mouse barely started shredding the nestlet resulting in a score of 2. (**c**) Mouse shredded half of the nestlet (Score=3). (**D**) Mouse almost shredded the entire nest (Score=4). (**e**) Mouse shredded the entire nestlet and built a fluffy nest resulting in a score of 5.*

2.7.3 Sucrose preference test

Anhedonia was tested in sucrose preference test. Anhedonia, or the decreased ability to feel pleasure, represents a key symptom of depression. Alterations in sucrose preference can be linked to different affective disorders and may occur from changes in CREB activity in the reward pathway consisting of the dopaminergic neurons extending from the ventral tegmental area to the nucleus accumbens (Barrot et al. 2002). Studies have shown that a decrease in sucrose preference can be reversed by treatment with tricyclic antidepressants (P. Willner et al. 1987). For sucrose preference test, mice were single housed and could freely choose between a water bottle and a bottle filled with 1% sucrose solution. In general, mice prefer the sweet sucrose solution to water up to 95% (Pothion et al. 2004). Sucrose preference was tested during 3 days by weighting of the bottles, the body weight and food intake of mice daily. The position of bottles was switched daily to exclude a side preference. Sucrose preference in percentage was calculated using the sucrose solution consumed divided by the total amount of liquid consumption.

2.7.4 Open-field test

Information about general motility, locomotor activity and anxiety of conditional Dagla KO mice was obtained in the open-field test. In this test, mice were allowed to move freely in an open-field box (44 cm x 44 cm) for 30 min. The boxes were moderately illuminated with a light intensity of around 130 lux. Time spent in center (25% of entire box) was considered as measure of anxiety, since mice normally try to avoid open, illuminated areas. Freezing behavior or decreased movement are also an indication for high anxiety. Analysis was executed using "Ethovision XT" software (Noldus Information Technology Inc). After each mouse, boxes were cleaned with ethanol to remove smell.

2.7.5 Zero-maze test

The zero-maze test was performed to test anxiety-related behavior. The zero-maze (40 cm height; 46 cm diameter) is divided into four equal quadrants, two open and two closed quadrants (Figure 2.4). The test is based on the knowledge that anxious mice try to avoid open areas completely, while normal mice prefer closed areas but explore unfamiliar areas as well. The maze was illuminated with a light intensity of around 200 lux in a sound-isolated room.
In the beginning of the test, mice were always placed into the same open quadrant and their movement was recorded for 5 min. After each test trial the zero-maze was cleaned with ethanol. Analysis of the time spent in different areas, distance travelled and velocity was executed with "Ethovision XT" software (Noldus Information Technology Inc).
Additionally, stretched postures while leaving the closed areas and looking-down behavior were counted according to Shepherd *et al.,* 1994 (Shepherd et al. 1994).
Looking-down behavior is an indication of low anxiety while stretched posture before leaving the closed area is an indication of higher anxiety and cautious behavior (Shepherd et al. 1994).

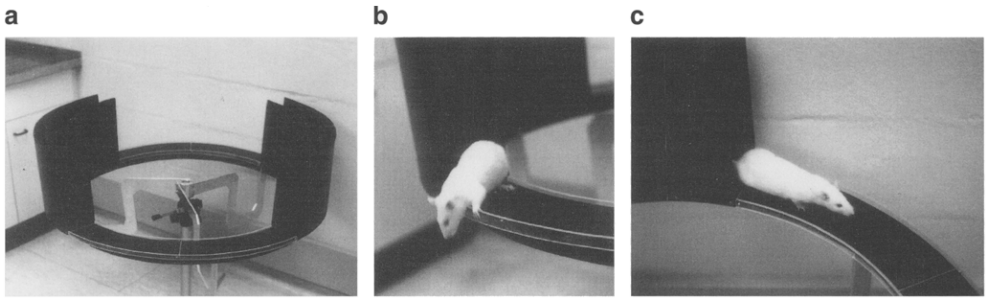


Figure 2.4: : Visualization of zero-maze construction, looking-down behavior and stretched posture (Shepherd et al. 1994).

(A) *O-Maze construction* ***(B)*** *Looking-down behavior, which is an indication for low anxiety* ***(C)*** *Stretched posture while leaving the closed area as an indication for cautious, anxious behavior.*

2.7.6 Forced swim test

The forced swim test, also known as ´Porsolt test´, is the most common test for depression-like behavior in mice. In this test, mice were placed in a glass cylinder (28 cm height, 20 cm diameter) filled with water (25-26 °C) for 6 minutes. In the last 4 minutes of the test, immobility time of mice was measured. Immobility time or floating behavior is interpreted as despair behavior, since mice have the natural instinct to escape from stressful stimuli like water. Additionally, immobility time can be reduced by antidepressants (Petit-Demouliere, Chenu, and Bourin 2005).

After the test, mice were dried with paper tissue. The entire test was recorded on video (Handycam).

2.7.7 Vision and olfaction tests

Vision and olfaction tests were performed to exclude that the observed phenotypes are caused by the inability of mice to see or to smell. For behavioral tests like pup retrieval test a proper vision and olfaction is necessary for good performance. Blindness could also lead to increased anxiety, which is estimated in tests like open-field or zero-maze. Since GFAP- and GLAST-Dagla KO mice showed a strong phenotype in some of these tests, we decided to perform the vision and olfaction test in those two lines. Moreover, GFAP and GLAST are expressed in the retina of mice (Jha et al. 2017; Lehre, Davanger, and Danbolt 1997).

For the vision test, mice were held at their tail base and were slowly moved down to the grid of their home cages from approximately 20 cm distance. Simultaneously mice´ forelimbs were observed. If the mouse stretched the forelimbs before whiskers were touching the grid, mice received a score of 3. If the mouse stretched them when whiskers were already touching the grid it received a score of 2. If the nose was touching the grid, it was scored with 1. Mice evaluated with a score of 3 are able to see.

For the olfaction test, mice were habituated to a cotton swab for 30 minutes in their home cages. Afterwards, a cotton swab dipped in water was offered to the mouse for 2 minutes. The time a mouse was spending actively with the cotton swab was measured. Subsequently another cotton swab dipped into the urine of a mouse from the other gender was offered to the mouse for 2 minutes and again the time the mouse was actively spending with the swab was measured. Mice spending more time with the cotton swab dipped in urine are able to smell (Zou et al. 2015).

2.7.8 Home-cage activity measurements

Activity and motility of mice were determined by home-cage activity measurements. Home cage activity of single housed mice was recorded using an infrared sensor which was attached to the cage lid. Movements in the home cage were recorded every 30 seconds and were analyzed by Mouse-E-Motion system (Infra-e-motion GmbH). Measurements shown in the graphs were summed up to 1 hour.

2.7.9 Social avoidance test

The social avoidance test was performed to assess social behavior/anxiety in case the results from other anxiety test were not straight-forward. For the test, mice were place in an open-field box (see 2.7.4.) containing a small cage with a partner mouse (C57BL6/J) from the other sex in one corner of the box. The open-field box was highly illuminated (~245 lux) and mice were allowed to move freely in the box for 5 min. The EthoVision software was used to record the movements of the mouse and to measure the time the test mouse was interacting with the partner mouse. If mice spent less time investigating the partner mouse, this behavior was defined as social avoidance behavior or social anxiety.

2.7.10 Light/dark test

The light/dark test was performed to assess anxiety behavior. For this test, an open-field box was divided into a light area and a dark area connected by a small hole. The box was placed into a sound-isolated room and the light area was highly illuminated (~1500 lux). Mice were always placed in the dark area in the beginning of the test and were allowed to move freely in the box for 10 min. Movements were recorded using the ActiMot System (TSE Systems). This test is based on the knowledge that mice usually avoid open and illuminated spaces. In case of anxiety, the time investigating the light area is lower than in control mice.

2.8 Targeted lipidomics (endocannabinoid measurements)

2.8.1 Determination of estrous cycle in female mice

Since endocannabinoid levels are fluctuating during the estrous cycle, female mice were checked for their cycle state before obtaining the brains. According to Bradshaw *et al.* (2006), changes in endocannabinoid levels in female rat brain have been predominantly observed during the 36h time period surrounding ovulation and behavioral estrus (also known as proestrus). Bradshaw *et al.* systematically measured the levels of anandamide, 2-arachidonoyl glycerol, N-arachidonoyl glycine, N-arachidonoyl gamma amino butyric acid, and N-arachidonoyl dopamine in seven different brain areas (pituitary, hypothalamus, thalamus, striatum, midbrain,

hippocampus, and cerebellum) in female rats at five different points in the estrous cycle. The cerebellum did not demonstrate a change in endocannabinoid production across the estrous cycle, whereas all other areas tested showed significant differences in at least one of the compounds during the proestrus (Bradshaw et al. 2006).

The estrous cycle state of female mice was estimated by visual observation of the vaginal opening (see Figure 2.5). For organ retrieval, only female mice in metestrus (C) or diestrus (D) were used. Mice in proestrus (A) or metestrus (B) were sacrificed at a later time-point.

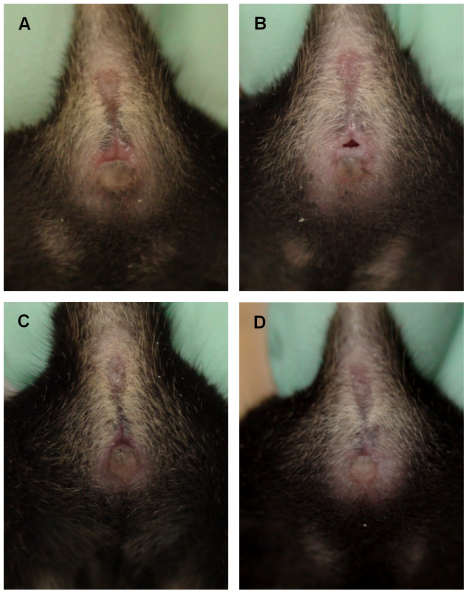


Figure 2.5: Anatomical differences in the female sex organ during estrous cycle.

The four stages of estrous (proestrus (A), estrus (B), metestrus (C), diestrus (D)) are shown for a Bl6C57 strain (Byers et al. 2012). *For endocannabinoid measurements, mice were killed in metestrus (C) or diestrus (D).*

2.8.2 Tissue preparation

For brain tissue preparation, mice were killed by decapitation. Immediately, blood was collected in EDTA tubes (Sarstedt) and stored at 4 °C. Liver and adrenal glands were collected in reactions tubes and frozen in dry ice. Brains were isolated and washed in cooled PBS. With the help of a brain matrix and punchers, hypothalamus, prefrontal cortex, striatum, hippocampus, amygdala and retrosplenial cortex were isolated in 3 minutes to prevent endocannabinoid degradation. Additionally, adrenal glands and liver were isolated for endocannabinoid measurements and molecular analysis. All tissues were frozen in dry ice and stored in -80 °C. After blood was collected–from every fourth mouse into EDTA tubes, tubes were centrifuged at 2000 *g* for 10 minutes to remove cells. The resulting supernatant was designated as plasma and transferred into a new reaction tube and stored at -80 °C.

2.8.3 Targeted lipidomics (endocannabinoid measurements)

Measurements of arachidonate lipids (endocannabinoids and their metabolites) were performed in collaboration with the Institute of Biochemistry and Molecular Medicine in Bern. Extraction of lipids and measurements were conducted by Sandra Glasmacher. Extraction and quantification of basal concentration of endocannabinoid system components was carried out using LC-MS/MS procedure as previously described by Lomazzo *et al.,* 2015 (Lomazzo et al. 2015).

2.9 Corticosterone measurements

2.9.1 Collection and processing of mouse feces

Feces were collected 24 h after the cages were changed, to measure an average level of corticosterone during one day. Per mouse, one 1.5 mL reaction tube of feces was collected with a pair of forceps.

Feces were transferred into a 35 mm Petri dish and dried in an oven at 50 °C for 1 h. In a mortar, feces were grinded as small as possible and 0.2 g of feces-powder were weighed and transferred into a 5 mL reaction tube. 2 mL of absolute ethanol were added and incubated for 30 min under shaking to dissolve proteins and release corticosterone. Subsequently, samples were centrifuged for 30 min at 4600 rpm at RT and the supernatant was transferred into a 2 mL reaction tube. In the SpeedVacuum centrifuge EtOH was evaporated at 35 °C for 1-1.5 h until a dry pellet was left. Extracted samples were stored at 20 °C overnight.

2.9.2 Corticosterone measurements by ELISA assay

Fecal corticosterone levels were measured using the Enzyme Immunoassay Kit (Arbor Assays). Therefore, extracted samples were dissolved in 100 µL EtOH and 400 µL assay buffer, vortexed and incubated for 5 min at RT. Vortexing and incubation were repeated two times. Afterwards, samples were diluted 1:5 with assay buffer to decrease ethanol concentration that needs to be lower than 5% for the immunoassay. Corticosterone standards were prepared according to the

manufacturer´s protocol (78,125 pg/mL – 10000,0 pg/mL). 50 µL of all samples and standards were pipetted in duplicates into the wells of a clear microtiter plate coated with an antibody to capture sheep antibodies. 25 µL of corticosterone-peroxidase conjugate was added to the standards and samples in the wells. The binding reaction is initiated by the addition of a polyclonal antibody to corticosterone to each well. After one hour of incubation at RT, the plate was washed four times with 300 µL of washing buffer. Afterwards, 100 µL of substrate were added to each well. The substrate reacts with the bound corticosterone peroxidase conjugate. After a short incubation of 30 min, 50 µL of stop solution were added to stop the reaction. The intensity of the generated color was analyzed at 450 nm in a microtiter plate reader (Dynex Technologies; MRX TC).

2.10 Programs

Graphs shown in this thesis were generated and analyzed using Graph Pad Prism 7.0d. Illustrative figures were designed using BioRender.

2.11 Statistics

Data are presented as means ± SEM. The number of animals/samples used is indicated in the figure legends. Statistical significance was assessed by Student *t*-test, Mann-Whitney test (for non-parametric data) or two-way ANOVA (Sidak post-hoc test). Significant outliers were identified and excluded using Grubbs´ test.

3 Results

3.1 Neuron-specific Syn-Dagla mice

3.1.1 DAGLa expression and endocannabinoid production by neurons

To investigate the role of DAGLa in neurons, we used conditional Syn-Dagla KO mice. *Dagla* deletion in this mouse model was first assessed by immunohistochemistry (Figure 3.1). Dagla fl/fl control mice showed a high DAGLa signal in hippocampus, cortex and thalamus, whereas the signal in hypothalamus and amygdala was weaker. Syn-Dagla KO mice showed a reduction of DAGLa staining in thalamus, amygdala as well as in dentate gyrus and CA3 region of hippocampus. DAGLa signal in CA1 and CA2 region was similar compared to Dagla fl/fl controls. Constitutive Dagla KO mice were used as a negative control for antibody specificity and did not show any DAGLa staining.

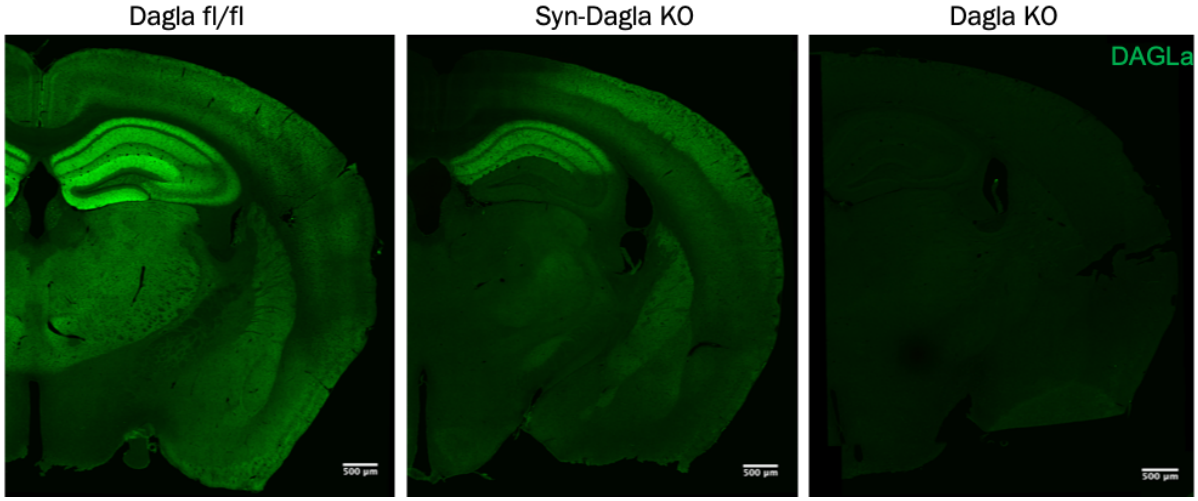


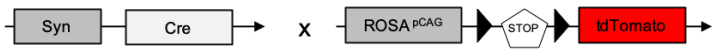
Figure 3.1 Validation of Syn-Dagla KO efficacy by immunohistochemistry

Representative images of DAGLa immunostainings from brains of Dagla fl/fl control mice, Syn-Dagla KO and constitutive Dagla KO control mice. DAGLa staining is completely absent in constitutive Dagla KO brains and reduced in some brain regions of Syn-Dagla KO mice, for example dentate gyrus and CA3 region of hippocampus. (Scale bar: 500 µm; green: DAGLa).

To further analyze the specificity and efficacy of the Syn promoter, we used the RosaTomato reporter line and fluorescent immunostainings with cell-type specific markers. In RosaTomato reporter mice, the fluorescent protein tdTomato is expressed in cells expressing Cre recombinase (Figure 3.2A). In hippocampus, Syn-Cre showed a high specificity for neurons (96.08-98.08%),

whereas promoter efficacy varied in the different areas of hippocampus. In dentate gyrus, 86.31% of neurons were tdTomato positive, while only 24.54% of neurons in CA1 showed tdTomato expression. In cingulate cortex, specificity of Syn-Cre for neurons was also relatively high but not many neurons expressed tdTomato. In hypothalamus, specificity and efficacy of the Syn-promoter were lower compared to the other tested regions. Additionally, confocal pictures (Figure 3.2C) showed a high tdTomato signal in the basolateral amygdala.

A



B

Syn-Dagla

Area	Promoter specificity (%)	Promoter efficacy (%)
Cingulate cortex	74.50 ± 14.45	17.67 ± 10.53
DG	96.08 ± 6.52	86.31 ± 11.10
CA3	96.33 ± 6.05	47.83 ± 23.07
CA1	98.08 ± 4.91	24.54 ± 9.78
Hypothalamus	37.83 ± 26.69	16.20 ± 9.67

C

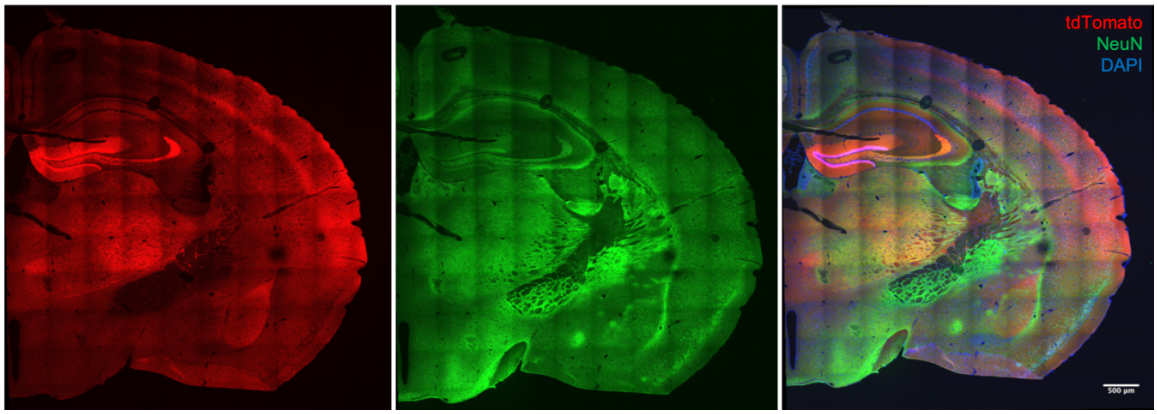


Figure 3.2 Validation of Cre expression in Syn-Dagla KO mice by Rosa-Tomato reporter line

(A) Genetic description of mice used for validation of Cre expression. (B) Quantification of approximate Cre expression specificity (% of cells co-expressing tdTomato and NeuN from all Cre-expressing cells) and efficacy (% of tdTomato cells co-expressing NeuN from all NeuN-positive cells) in different brain regions of Syn-RosaTomato mice. (C) Representative immunohistochemistry picture showing tdTomato expression in Syn-Cre line. Green shows neuronal-marker NeuN, tdTomato is shown in red (scale bar: 500 um). Values represent mean ±SD. n=3 per genotype. Rosa-Tomato reporter analysis (Table) was performed by PD Dr. Andras Bilkei-Gorzo, University of Bonn.

To uncover the contribution of neurons in the 2-AG production, targeted lipidomics was conducted in Syn-Dagla KO mice and their littermate controls to measure the levels of endocannabinoids and related lipids. Mice were decapitated and brain regions were dissected in less than 3 min to avoid enzyme degradation. Female mice were controlled for their estrus cycle, since endocannabinoid levels are changing in some brain areas during the estrus. Samples of different brain regions were

frozen in dry ice and sent to University of Bern for targeted lipidomics using an adapted LC-MS/MS procedure.

Even though neurons are believed to be main producers of the endocannabinoid 2-AG, the levels of 2-AG were unchanged in every tested brain region of both male and female Syn-Dagla KO mice compared to the littermate controls (Figure 3.3B). In contrast, levels of the DAGLa substrate 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) were significantly elevated in hippocampus of both male and female Syn-Dagla KO mice (Figure 3.3A). Levels of the metabolite of 2-AG, arachidonic acid (AA), were decreased in prefrontal cortex (PFC) and hippocampus of male Syn-Dagla KO mice compared to controls. In female Syn-Dagla KO mice, levels of AA were decreased in PFC, striatum and hippocampus, but not in hypothalamus (Figure 3.3C). It is noteworthy that 2-AG levels in general are lower compared to SAG or AA levels.

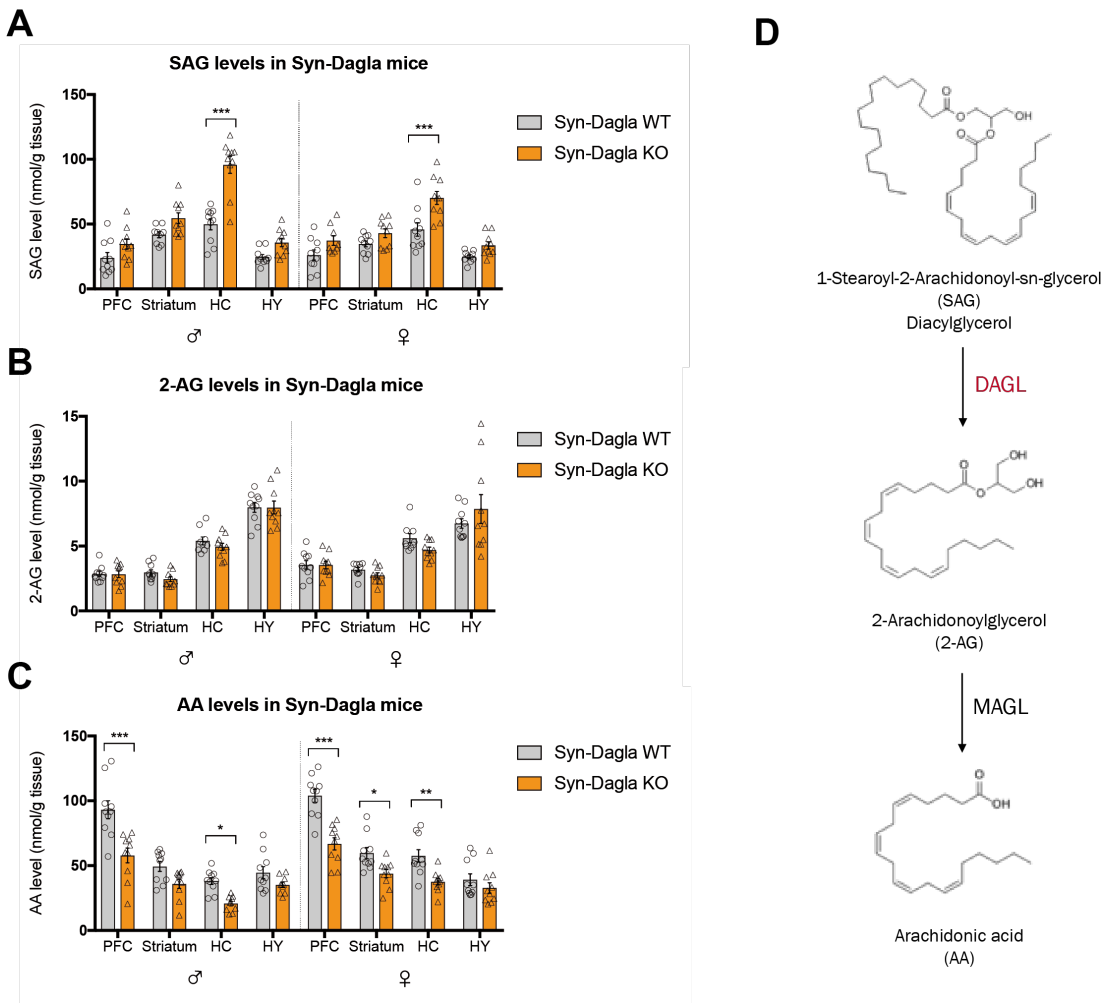


Figure 3.3: Measurements of components of the endocannabinoid system in Syn-Dagla mice by LC-MS/MS.

(A) *The substrate of DAGLa, 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), was significantly increased in hippocampus (HC) of male and female Syn-Dagla KO mice (males: genotype effect: $F_{1,17}$=49.91, p<0.0001; brain region effect: $F_{3,51}$=48.49, p<0.0001; interaction: $F_{3,51}$=7.372, p=0.0003; females: genotype effect: $F_{1,17}$=17.7, p=0.0006; brain region effect: $F_{3,51}$=27.64, p<0.0001).* ***(B)*** *Male and female Syn-Dagla KO mice did not show any significant reduction in 2-arachidonoly glycerol (2-AG) levels in any of the tested brain regions (prefrontal cortex (PFC), striatum, hippocampus (HC) or hypothalamus (HY)).* ***(C)*** *Arachodonic acid (AA) levels were significantly reduced in PFC and HC of male Syn-Dagla KO mice (genotype effect: $F_{1,18}$=19.16, p=0.0004; brain region effect: $F_{3,54}$=68.07, p<0.0001; interaction: $F_{3,54}$=5.608, p=0.002). In female Syn-Dagla KO mice AA levels were significant*

lower in PFC, striatum and hippocampus compared to controls (genotype effect: $F_{1,18}$=32.69, p<0.0001; brain region effect: $F_{3,54}$=57.14, p<0.0001; interaction: $F_{3,54}$=5.274, p=0.0029) ***(D)*** *Schematic representation of the synthesis and metabolism of 2-AG by the enzymes diacylglycerol lipase (DAGL) and monoacylglycerol lipase (MAGL). Values represent mean ± SEM; n=10 animals/group. Measurements were performed by Sandra Glasmacher, University of Bern.*

Prostaglandins are involved in inflammatory processes and represent a metabolite of 2-AG (Figure 3.4C). Prostaglandin E2 (PGE2) was significantly decreased in the PFC, striatum and hippocampus of male Syn-Dagla KO mice, whereas female Syn-Dagla KO mice only showed a significant decrease of PGE2 in the PFC compared to control mice (Figure 3.4A). Levels of another prostaglandin, prostaglandin D2, were also decreased in prefrontal PFC and striatum of male Syn-Dagla KO mice compared to littermate controls. In female mice, PGD2 was only significantly decreased in PFC in comparison to control mice.

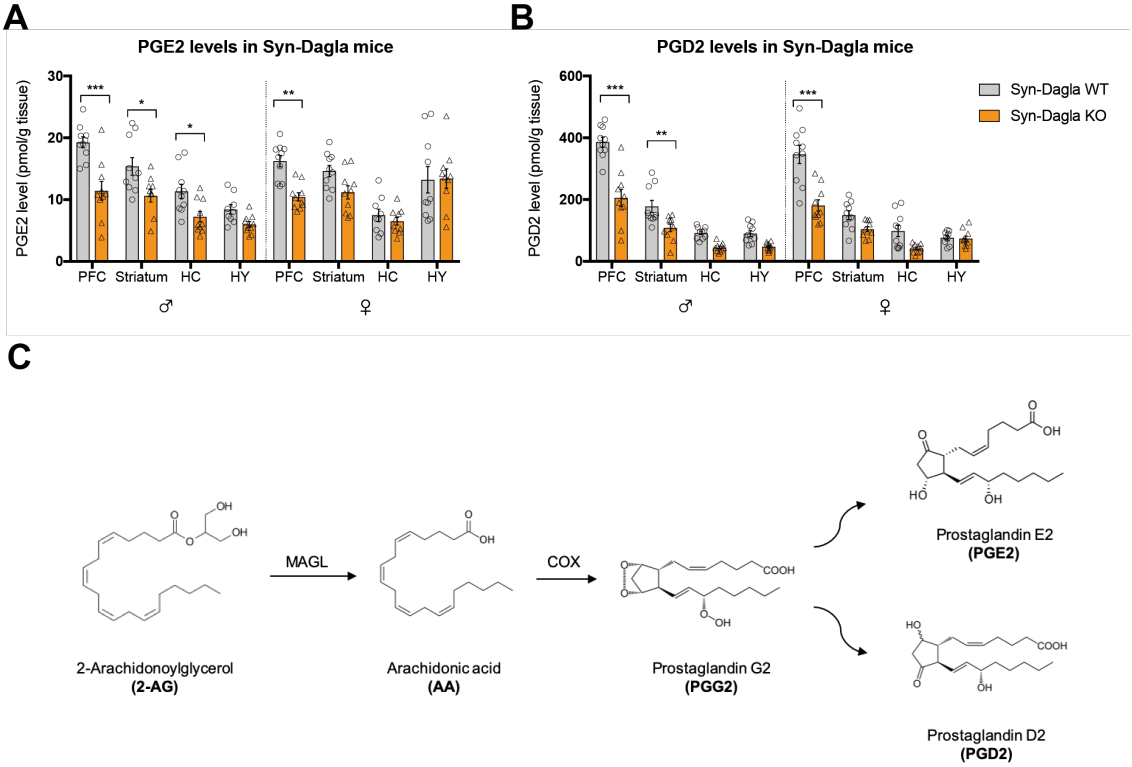


Figure 3.4: Measurements of prostaglandins in Syn-Dagla KO mice by LC-MS/MS.

(A) *Prostaglandin E2 (PGE2) levels were significantly reduced in prefrontal cortex (PFC), striatum and hippocampus (HC) of male Syn-Dagla KO mice compared to controls, but not in hypothalamus (HY) (genotype effect: $F_{1,17}$=16.68, p=0.0010; brain region effect: $F_{3,51}$=37.76, p<0.0001; interaction: $F_{3,51}$=3.616, p=0.0192). In female Syn-Dagla KO mice PGE2 levels were significantly reduced in PFC compared to littermate controls (genotype effect: $F_{1,17}$=4.731, p=0.0440; brain region effect: $F_{3,51}$=17.67, p<0.0001; interaction: $F_{3,51}$=2.843, p=0.0468).* ***(B)*** *Prostaglandin D2 (PGD2) levels were significantly lower in hippocampus and striatum of male Syn-Dagla KO mice compared to their littermate controls (genotype effect: $F_{1,18}$=31.69, p<0.0001; brain region effect: $F_{3,54}$=173.7, p<0.0001; interaction: $F_{3,54}$=15.8, p<0.0001). In female Syn-Dagla KO mice PGD2 levels were significantly reduced only in PFC compared to controls (genotype effect: $F_{1,17}$=13.68, p=0.0018; brain region effect: $F_{3,51}$=107.9, p<0.0001; interaction: $F_{3,51}$=15.07, p<0.0001).* ***(C)*** *Schematic representation of synthesis of prostaglandins E2 and D2 from 2-AG. Values represent mean ± SEM; n=10 animals/group. Measurements were performed by Sandra Glasmacher, University of Bern.*

Previously it was shown that constitutive Dagla KO mice also show reductions in the levels of the second endocannabinoid anadamide (AEA), which is actually produced by another enzyme: NAPE-PLD (Y. Gao et al. 2010; Jenniches et al. 2016a; Schurman et al. 2019). For this reason, we also measured AEA levels in our neuron-specific Dagla KO mice (Figure 3.5). However, AEA levels were unchanged in both, male and female Syn-Dagla KO mice.

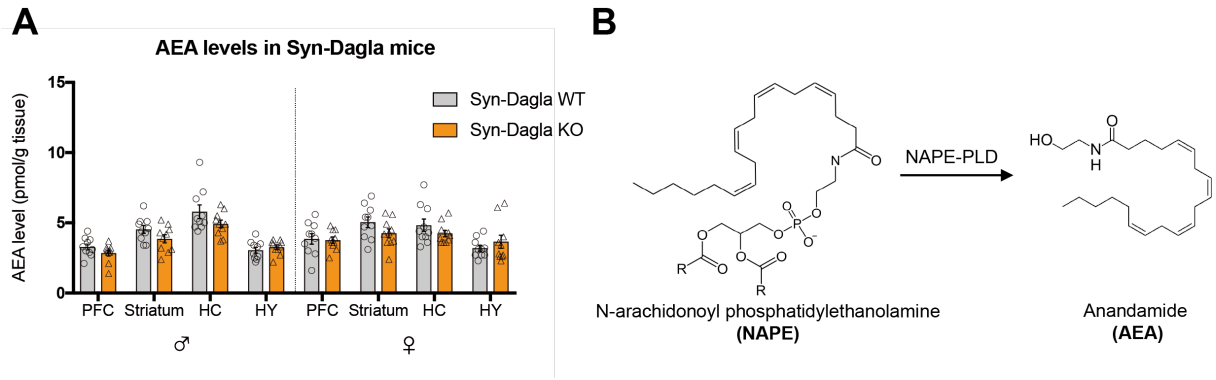


Figure 3.5: Measurement of the endocannabinoid anandamide in Syn-Dagla mice by LC-MS/MS.

***(A)** Levels of the second endocannabinoid anandamide (AEA) were unchanged in male and female Syn-Dagla KO mice in comparison to littermate controls (males: genotype effect: $F_{1,18}$=3.717, p=0.0698; brain region effect: $F_{3,54}$=33.36, p<0.0001; females: genotype effect: $F_{1,18}$=0.6876, p=0.4179; brain region effect: $F_{3,54}$=7.245, p=0.0004). **(B)** Schematic representation of synthesis of the endocannabinoid anadamide from N-Arachidonoyl phosphatidylethanolamine (NAPE) by the enzyme N-acetylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD). Values represent mean ± SEM; n=10 animals/group. Measurements were performed by Sandra Glasmacher, University of Bern.*

Summarized, Syn-Dagla KO mice show a very specific deletion of *Dagla* in neurons but a low efficiency of the promoter that only affects 16-86% of all neurons depending on the brain region. Even though neurons are believed to be the main producers of 2-AG, 2-AG levels were not changed in Syn-Dagla KO mice. Nevertheless, we measured an increase in the levels of DAGLa substrate, SAG, and a decrease in AA and PGs, the of metabolites 2-AG.

3.1.2 Body weight, food intake and survival of Syn-Dagla KO mice

Male Syn-Dagla KO mice showed a significantly reduced body weight (Figure 3.6A) and food intake (Figure 3.6B) compared to their WT littermates. This phenotype was even more pronounced in female Syn-Dagla KO mice (Figure 3.6C/D) which showed a strongly reduced food intake and body weight. In male mice, the bodyweight differences equaled out the older the mice became. During sucrose preference test, male Syn-Dagla KO mice and their controls seem to have adapted to additional caloric intake and reduced their food intake. In female mice, only WT controls reduced

their food intake during sucrose preference test, whereas Syn-Dagla KO mice kept on eating similar amounts as before.

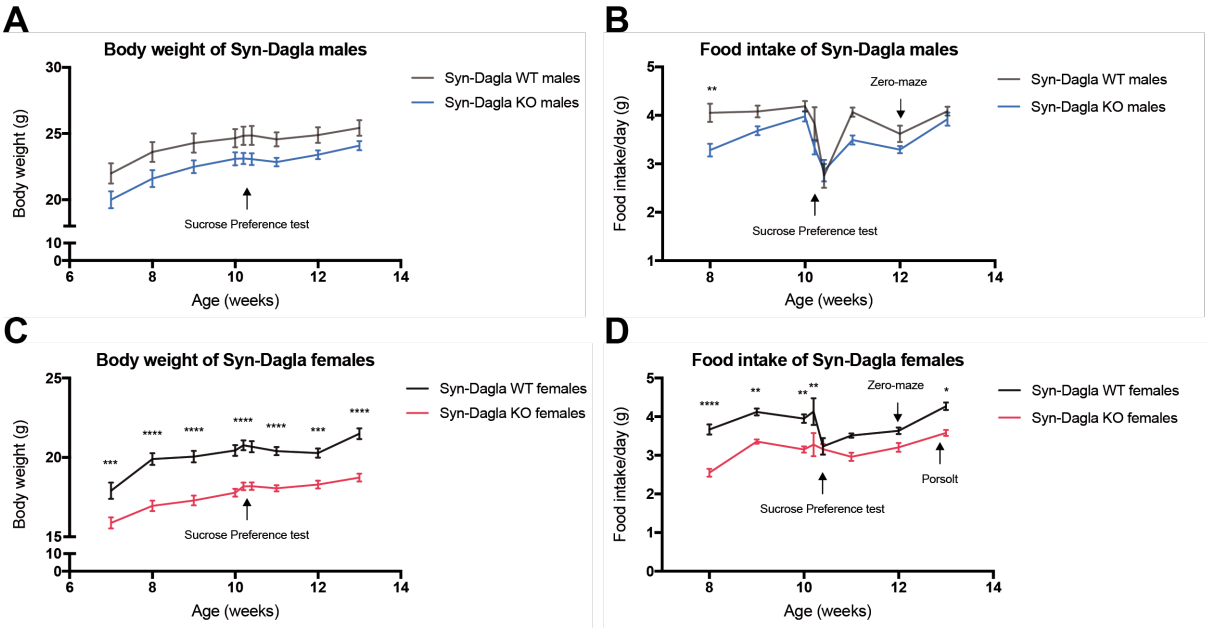


Figure 3.6: Body weight and food intake of male and female Syn-Dagla KO mice.

(A) *Body weight of male Syn-Dagla KO mice was significantly reduced compared to WT controls (genotype effect: $F_{1,18}$=4.695, p=0.0439; time effect: $F_{8,144}$=80.17, p<0.0001).* ***Post hoc*** *analysis was not significant for any of the tested time points.* ***(B)*** *Syn-Dagla KO mice show significantly reduced food intake compared to WT controls (genotype effect: $F_{1,18}$=6.723, p=0.0184; time effect: $F_{7,126}$=16.67, p<0.0001). In the post-hoc analysis, the first food intake measurement was significantly different between 8 weeks old KO and WT mice (p=0.0082). During sucrose preference test both, Syn-Dagla KO and WT, adapted to caloric changes and reduced their food intake.* ***(C)*** *Female Syn-Dagla KO showed a significantly reduced body weight in all measurements (genotype effect: $F_{1,18}$=41.3, p<0.0001; time effect: $F_{8,144}$=62.82, p<0.0001).* ***(D)*** *Food intake of female Syn-Dagla KO mice was significant lower during most of the measurements (genotype effect: $F_{1,18}$= 35.87, p<0.0001; time effect: $F_{7,126}$=8.417, p<0.0001). During sucrose preference test, only Syn-Dagla WT animals adapted to caloric changes by reducing their food intake, whereas Syn-Dagla KO mice kept eating a similar amount of food. Values represent mean ± SEM; n=10 animals/group.*

Astrogliosis is highly connected with brain injury or inflammation (Sofroniew 2015). It has also been shown that abnormalities in astrocytes occur during depression pathogenesis (Cotter et al. 2002; Nagy et al. 2015; Torres-Platas et al. 2011). Since 2-AG has anti-inflammatory properties and constitutive Dagla KO mice showed depression-related behavior, we analyzed the area covered with the astrocyte marker GFAP in the dentate gyrus of our mouse model. Syn-Dagla KO mice showed a significantly smaller area covered with GFAP in the dentate gyrus compared to their littermate controls in the two-way-ANOVA analysis. When controlling for multiple comparisons only one area of the dentate gyrus, the polymorph layer, showed a significant reduction in percentage of the area covered with GFAP (Figure 3.7B), whereas the number of astrocytes was not changed in dentate gyrus or polymorph layer (Figure 3.7C). Despite astrogliosis, considerable alterations in lipid levels and a possible epileptic profile as in constitutive Dagla KO mice, Syn-Dagla KO mice

showed normal survival (data not shown). During the behavioral testing phase, none of the mice died.

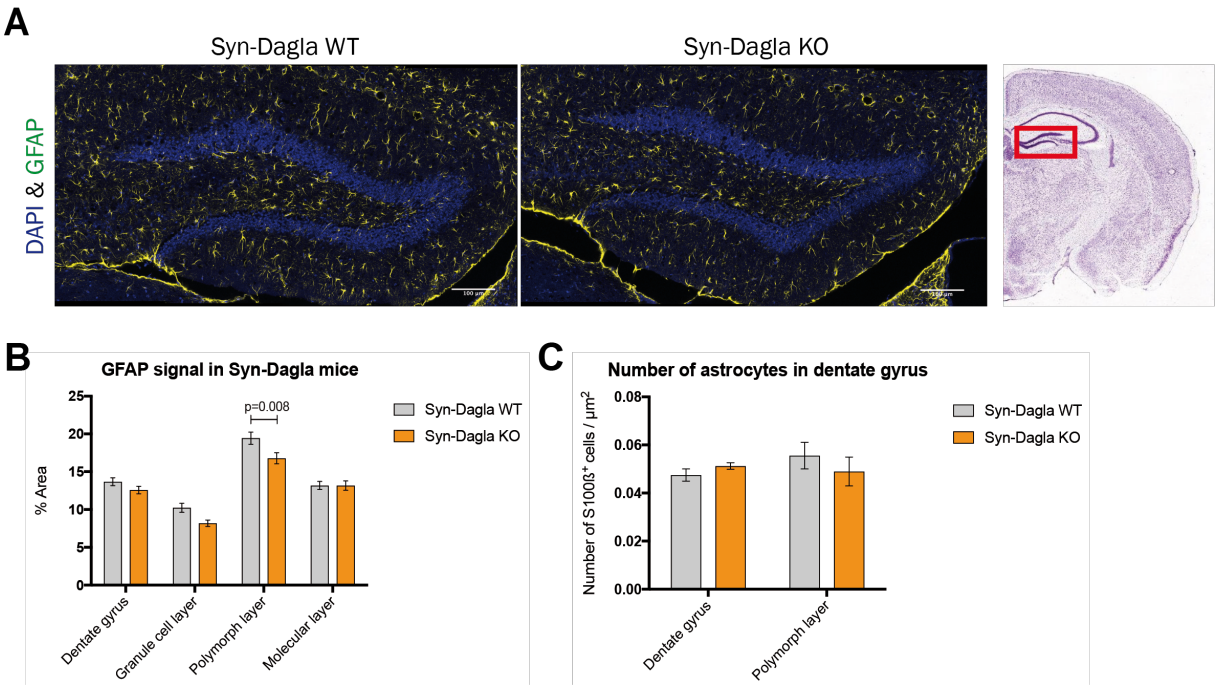


Figure 3.7: Analysis of astrogliosis in the dentate gyrus of Syn-Dagla KO mice and general survival of Syn-Dagla KO mice during the behavioral testing phase.

(A) *Representative immunohistochemistry pictures of a GFAP staining in the dentate gyrus of Syn-Dagla KO mice and control mice (scale bar: 100um).* ***(B)*** *Analysis of area covered with GFAP in different areas of dentate gyrus and entire dentate gyrus (genotype effect: $F_{1,172}$=11.79, p=0.0007; region effect: $F_{3,172}$=74.26, p<0.0001).* ***(C)*** *Number of astrocytes in the dentate gyrus of Syn-Dagla mice was analyzed using an S100β immunostaining. Six pictures were analyzed per animal and 4 animals were used per genotype (plotted: analysis of each picture).*

Taken together, Syn-Dagla KO mice show a decreased body weight and food intake compared to control mice. This phenotype is more pronounced in female mice, while the body weight differences in male mice equaled out during aging. Syn-Dagla KO mice show normal survival but a change in astrocyte morphology in the dentate gyrus, particularly in the polymorph layer (hilus).

3.1.3 Exploratory and anxiety-related behavior in Syn-Dagla KO mice

To assess locomotor activity in Syn-Dagla KO mice, home cage activity was measured for three days (Figure 3.8). Male and female Syn-Dagla KO mice showed significantly reduced activity in their home cages. Especially during the dark phase, in which mice are active, a strong reduction in the locomotor activity of Syn-Dagla KO mice was observed (Figure 3.8B/D), while activity was not significantly changed during the light phase.

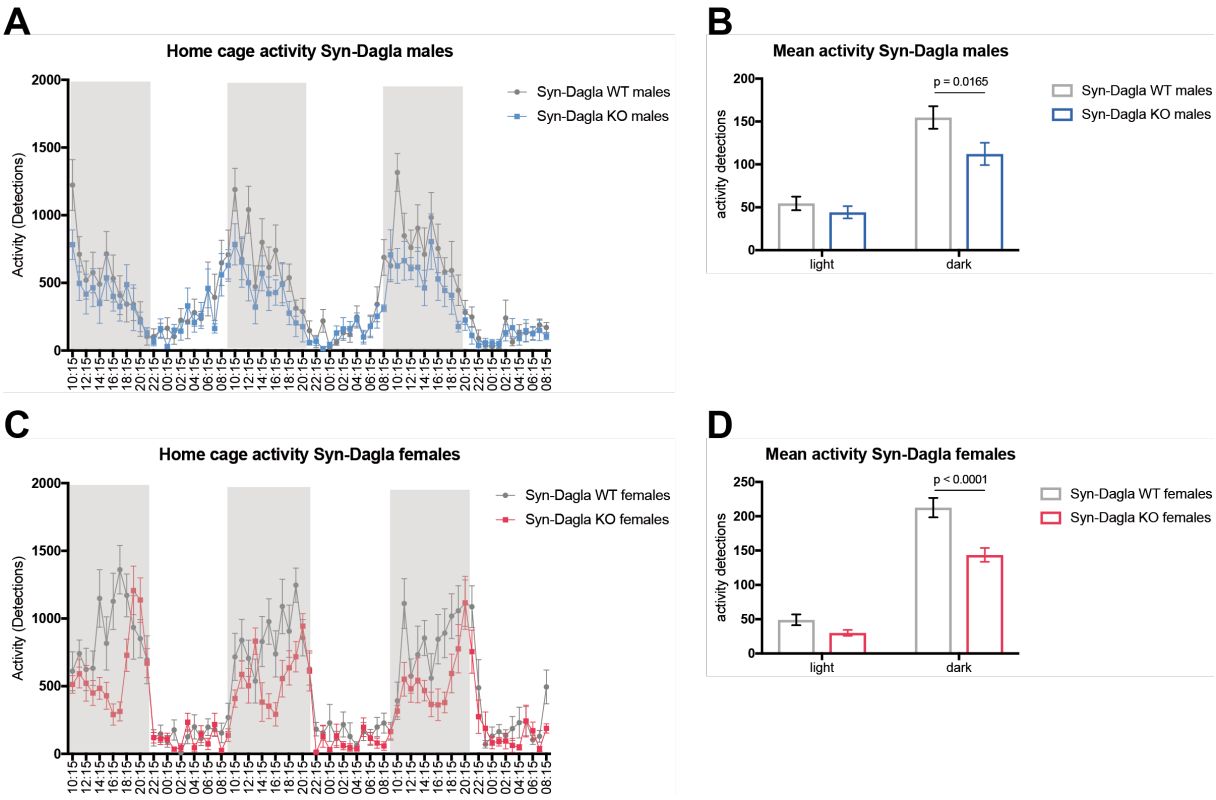


Figure 3.8 Home cage activity measurements of Syn-Dagla KO mice

(A) *Home cage activity measurements of male Syn-Dagla mice showed significantly reduced locomotor activity of Syn-Dagla KO mice compared to controls (genotype effect: $F_{1,17}$=15.17, p=0.0012; time effect: $F_{70,1190}$=10.84, p<0.0001).* ***(B)*** *Activity during the light-phase did not differ between male Syn-Dagla KO mice and male controls genotypes, whereas activity was significantly changed during the dark active phase.* ***(C)*** *Female Syn-Dagla KO mice showed a significant reduction in locomotor activity in their home cages compared to control mice (genotype effect: $F_{1,18}$=13.79, p=0.0016; time effect: $F_{70,1260}$=16.84, p<0.0001).* ***(D)*** *There were no changes in activity during the light-phase, whereas activity of female Syn-Dagla KO mice was significantly reduced during the dark active phase. Values represent mean ± SEM; n=10 animals/group.*

In the open-field test (Figure 3.9A/B), male Syn-Dagla KO mice spent significantly more time in the center of the box and showed a slightly reduced distance traveled compared to their WT littermates, which can be interpreted as reduced anxiety. Female Syn-Dagla KO mice did not show significant changes in exploratory activity in the open-field. In zero-maze test, male Syn-Dagla KO mice spent more time in the open compartments of the maze and moved a higher distance during the five minutes of testing. This phenotype as well points towards a rather “brave” behavior. Additionally, velocity of male Syn-Dagla KO mice in the closed compartments of the maze was increased compared to their littermate controls. Female Syn-Dagla KO mice showed similar tendencies but no significant changes in any of these parameters (Figure 3.9C-E). Both, male and female Syn-Dagla KO mice showed decreased execution of “stretched posture” and were looking down from the maze significantly more often than their WT littermates (Figure 3.9G/H), indicating a reduced anxiety according to Shepard *et al.* (Shepherd et al. 1994).

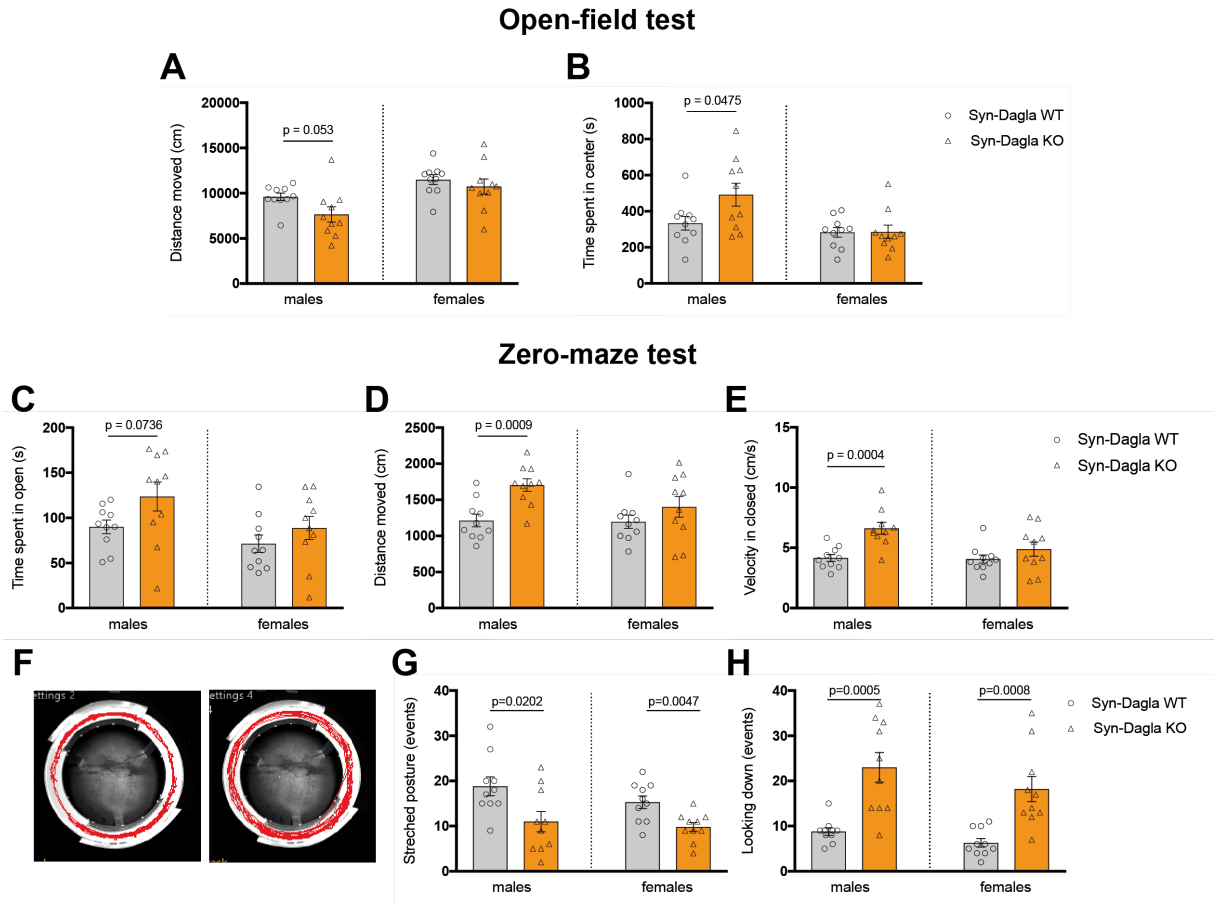


Figure 3.9: Exploratory- and anxiety-related behavior of Syn-Dagla KO mice.

***(A)** In the open-field test male but not female Syn-Dagla KO mice showed a tendency toward a reduction of distance traveled compared to WT controls. **(B)** Time spent in the center of the open-field box was increased in male, but not in female Syn-Dagla KO mice. **(C)** In zero-maze test male but not female Syn-Dagla KO mice spent slightly more time in the open compartments of the maze compared to WT controls. Furthermore, male Syn-Dagla KO mice traveled a higher distance in the maze **(D)** and showed higher velocity in the closed compartments **(E)**. Female Syn-Dagla KO mice did not show any significant differences in distance traveled or velocity **(D,E)**. **(F)** Representative tracking maps of male Syn-Dagla WT (left) and KO (right) mice show the route of one individual mouse in the zero-maze during five minutes. **(G)** Male and female Syn-Dagla KO mice displayed a stretched posture before leaving the closed compartment of the maze significantly less often than WT controls, while they were looking down from the maze significantly more often than the WT controls **(H)**. Values represent mean ± SEM; n=10 animals/group. Student´s t-test.*

Surprisingly, it seemed like Syn-Dagla KO mice were increasing their activity during the zero-maze test (distance and velocity), even though they showed generally decreased locomotor activity in their home cages (Figure 3.8). Probably this was a result of the novelty of the situation and is connected to stress. Thus, we measured if the levels of the stress hormone corticosterone (CORT) were generally changed in Syn-Dagla KO mice. Therefore, feces of Syn-Dagla KO mice and controls were collected one week after single housing and 24 hours after changing of the cages. Here, we wanted to investigate the baseline CORT levels (24h mean) of unstressed Syn-Dagla KO mice and controls (Figure 3.10B). CORT was extracted from the feces and an ELISA was performed to measure the CORT concentration. There were no changes in baseline 24h mean CORT concentration in male or female Syn-Dagla KO mice compared to controls. One day after the forced swim test, feces were collected again to measure the 24h mean CORT levels of stressed mice

(Figure 3.10C). Again, no differences were measured in the ELISA. When animals were killed for endocannabinoid measurements after the entire behavioral cassette, CORT levels were measured again in different brain regions of the mice by LC-MS/MS (Figure 3.10A). In these measurements, samples were taken at a single time point, opposing to the ELISA experiment, where the average of CORT fluctuations during 24 h was measured. CORT levels in the brain are mimicking the CORT levels in the blood since CORT is diffusing from blood to all tissues. After killing the mice, a significant increase in CORT levels was measured in the brain in both, male and female mice. This change was not significant only in the hippocampus of male mice in the post-hoc test. In general, the increase of CORT was higher in female than in male mice.

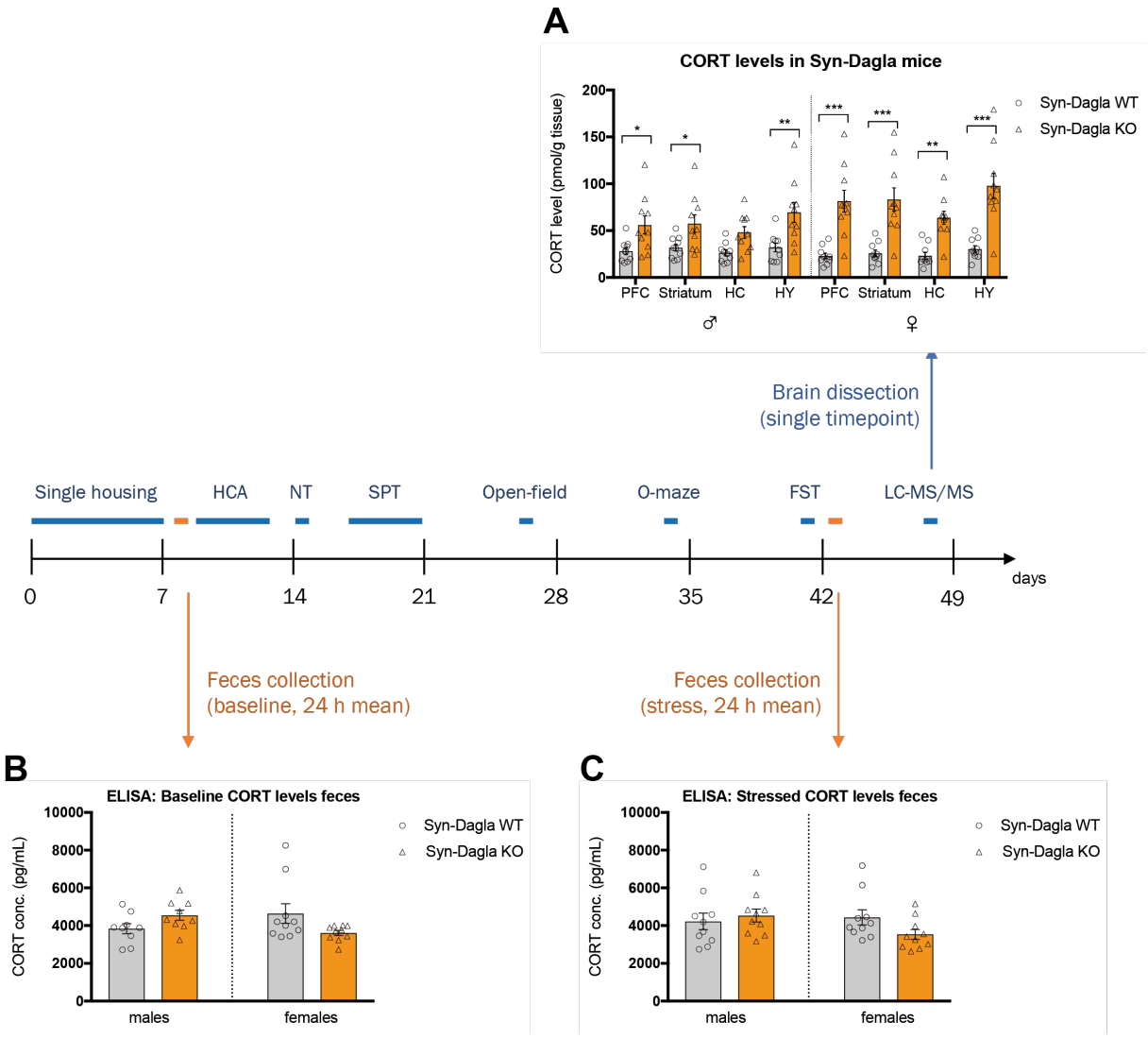


Figure 3.10: Corticosterone (CORT) measurements in Syn-Dagla KO mice by LC-MS/MS and ELISA.

(A) After animals went through all behavioral test, mice were decapitated and CORT levels were measured in different brain areas by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Male Syn-Dagla KO mice showed highly elevated CORT levels in prefrontal cortex (PFC), striatum and hypothalamus (HY) (Genotype effect: $F_{1,18}$=8.652, p=0.0087; brain region effect: $F_{3,54}$=19.12, p<0.0001; interaction: $F_{3,54}$=6.686, p=0.0006). In female Syn-Dagla KO mice, CORT levels were elevated in all tested brain regions (Genotype effect: $F_{1,18}$=24.34, p=0.0001; brain region effect: $F_{3,54}$=19.82, p<0.0001, interaction: $F_{3,54}$=8.676, p<0.0001). (B) Overall changes in CORT rhythm were measured from feces extract by CORT ELISA. Feces were collected after one week of single housing to measure 24 h mean CORT levels in unstressed animals. Neither in male nor in female Syn-Dagla KO

*mice, changes in Cort levels were detected in comparison to littermate controls. **(C)** 24 hours after the forced swim test, feces were collected again to measure CORT levels in stressed animals by CORT ELISA. There were no differences in 24 h mean CORT levels in male and female Syn-Dagla KO mice in comparison to their littermate controls. Values represent mean ± SEM; n=10 animals/group.*

Since both tests to evaluate anxiety-related behavior showed a phenotype of rather reduced anxiety, and at the same time elevated stress levels were measured in Syn-Dagla KO mice, we decided to add two additional tests to assess anxiety-related behavior. We performed a social anxiety test and the dark/light test. In the social avoidance test, male but not female mice showed a significantly reduced interaction with a partner, pointing towards a social avoidance or social anxiety (Figure 3.11A). Furthermore, female Syn-Dagla KO mice showed a smaller distance moved during the test, compared to the female control mice (Figure 3.11B). In the dark/light test, male and female Syn-Dagla KO mice spent significantly more time in the bright area of the test box, suggesting a decreased anxiety. The general distance the mice moved in the box was unchanged in both genders, while the distance moved in the light area of the box was significantly higher in Syn-Dagla KO mice and significantly reduced in the dark area (Figure 3.11D,E,F). Moreover, number of rearings was reduced in female Syn-Dagla KO mice compared to controls, suggesting decreased exploration.

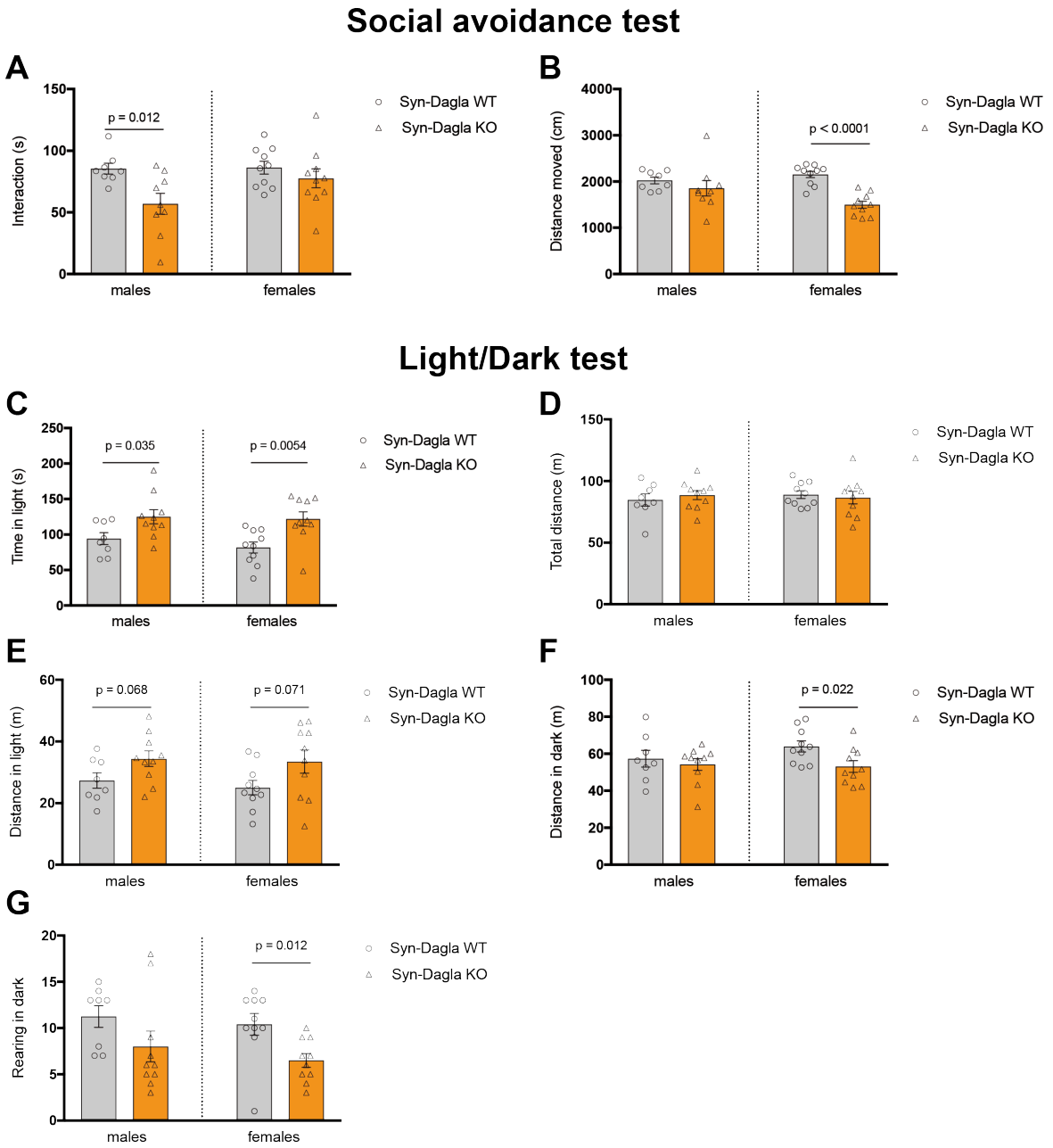


Figure 3.11: Anxiety-related behavior of Syn-Dagla KO mice assessed by social avoidance test and light/dark test.

(A) In the social avoidance test, male Syn-Dagla KO mice showed decreased interaction time with the partner mouse compared to male littermate controls. (B) Female Syn-Dagla KO mice showed significantly decreased distance moved during the social avoidance test. (C) In the light/dark test, male and female Syn-Dagla KO mice spent more time in the light area, compared to control littermates. (D) Total distance moved was unaltered, whereas distance moved in the light compartment was significantly higher in Syn-Dagla KO mice compared to the littermate controls (E). In the dark compartment, female Syn-Dagla KO mice moved less (F) and number of rearings was significantly reduced compared to controls (G). Values represent mean ± SEM; n=10 animals/group. Student´s t-test.

Our results indicate changes in exploratory and anxiety-related behavior in mice lacking DAGLa in neurons. Syn-Dagla KO mice showed a decreased locomotor activity in the home cage. In open-field and zero-maze, male Syn-Dagla KO mice showed braver behavior than their control mice. In zero-maze, Syn-Dagla KO mice additionally displayed increased locomotor activity, opposing to the decreased activity measured in the home cage. In the social avoidance test, male Syn-Dagla KO

mice showed higher social anxiety, while anxiety of Syn-Dagla KO mice in the dark/light test, on the other hand, seemed to be reduced. Additionally, Syn-Dagla KO mice displayed strongly increased CORT levels after acute stress, while the 24h mean measurements of CORT showed no differences between Syn-Dagla KO mice and controls.

3.1.4 Depression-like behavior in Syn-Dagla mice

Depression-like behavior was assessed in the nestlet, sucrose-preference and forced swim tests. In the nestlet test, male and female Syn-Dagla KO mice shredded a similar amount of nestlet compared to their littermate controls in five hours. After 24h, the nests of male and female mice were evaluated again using scores according to Deacon *et al.*, 2006. Syn-Dagla KO and control mice of both genders build a similar quality of nests also after 24 h.
Since food intake and body weight were reduced in Syn-Dagla KO mice, we assumed that Syn-Dagla mice might also drink less compared to their controls. Thus, we measured general water consumption of Syn-Dagla KO mice before starting the sucrose preference test (Figure 3.12C). Both male and female Syn-Dagla KO mice were drinking significantly less water in three days compared to their WT littermates. Even though consumption of liquid was reduced in Syn-Dagla mice, which was also the case during the sucrose preference test, Syn-Dagla KO mice preferred 1% sucrose solution over water. Syn-Dagla KO mice did not display an anhedonia phenotype in the sucrose preference test. In the forced swim test (Figure 3.12E), female Syn-Dagla KO mice showed a significantly elevated immobility time compared to their WT littermates. Male Syn-Dagla KO mice showed a similar immobility time as their controls. It is noteworthy that male Syn-Dagla KO mice did not show changes in body weight and food intake before the forced swim test, whereas female Syn-Dagla KO mice showed a highly reduced body weight and food intake before the test. It is plausible that the lack of energy in females might have influenced the forced swim test results.

Altogether, we did not observe depression-like behavior in Syn-Dagla KO mice. Only elevated immobility time of female Syn-Dagla KO mice might indicate a mild depression-like phenotype.

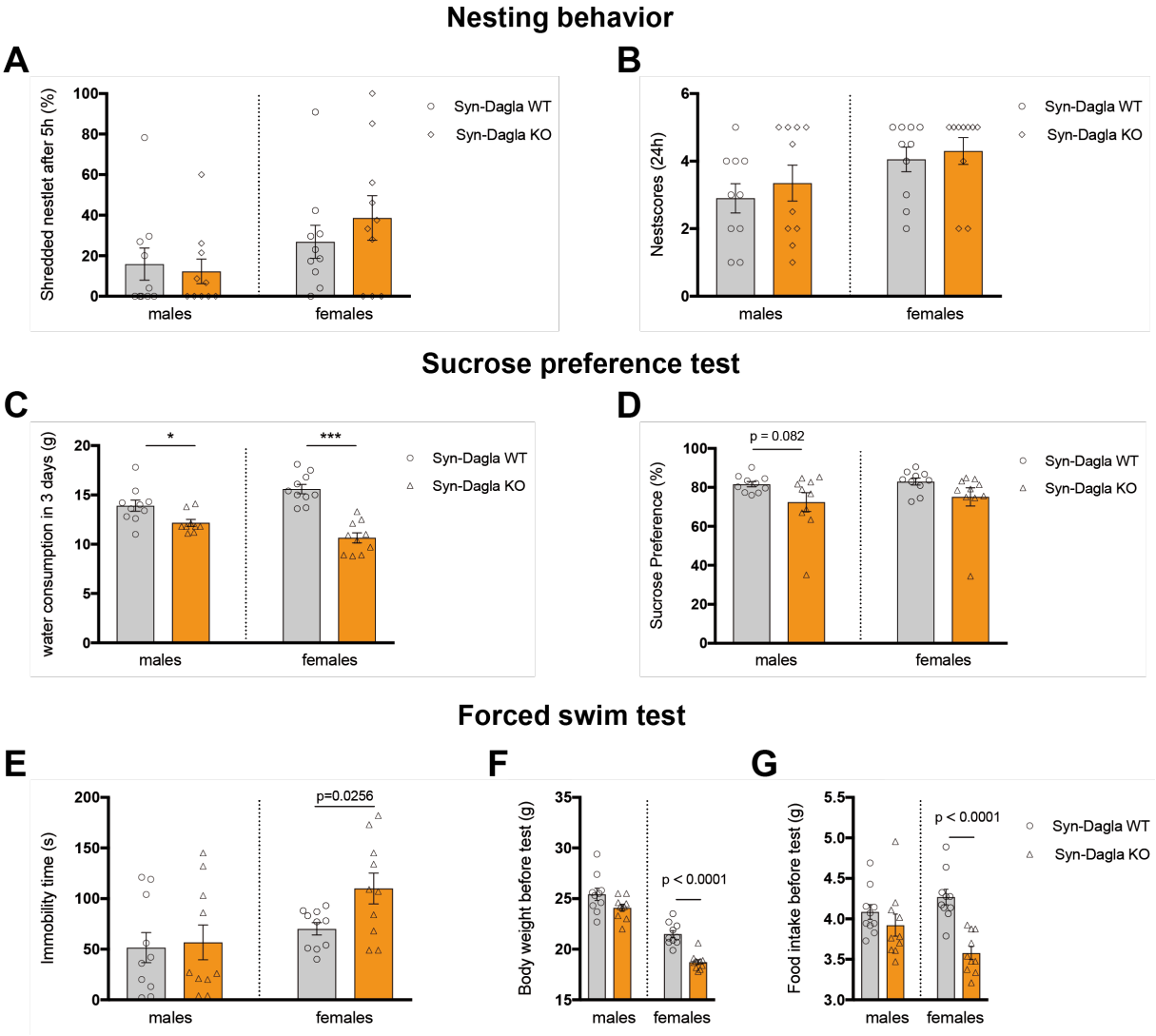


Figure 3.12 Depression-like behavior in Syn-Dagla KO mice.

(A, B) Nest-building behavior was not changed in neither male nor female Syn-Dagla KO mice. (A) In 5 h, both genotypes shredded similar amounts of the nestlet. (B) After 24 h, Syn-Dagla KO and control mice also received similar nest scores. (C, D) Sucrose preference was unchanged in male and female Syn-Dagla KO mice compared to controls. (C) In general, Syn-Dagla KO mice were drinking significantly less water in three days. In sucrose preference test Syn-Dagla KO mice were also drinking less liquid but they preferred sucrose over water like WT controls (D). (E) In the forced swim test male Syn-Dagla KO mice did not show differences in immobility time compared to their controls, whereas female Syn-Dagla KO mice showed significantly higher immobility time compared to controls. (F) Body weight measurements directly after the test showed that female Syn-Dagla KO mice had a significantly lower body weight compared to their littermate controls, while the body weight of male mice did not differ. (G) The food intake in the week before the test was also significantly reduced only in female Syn-Dagla KO mice. Values represent mean ± SEM; n=10 animals/group.

3.1.5 Maternal care behavior of Syn-Dagla mice

Next, we investigated maternal care and depression-like behavior in experienced Syn-Dagla KO dams in the pup retrieval test (Figure 3.13A). All tested Syn-Dagla KO dams retrieved three of their pups in less than two minutes, similar to Dagla fl/fl control dams, suggesting normal maternal care

behavior. Additionally, we observed milk spots in pups of Dagla fl/fl control dams and Syn-Dagla KO dams, an indication of normal breast-feeding and suckling behavior of pups.

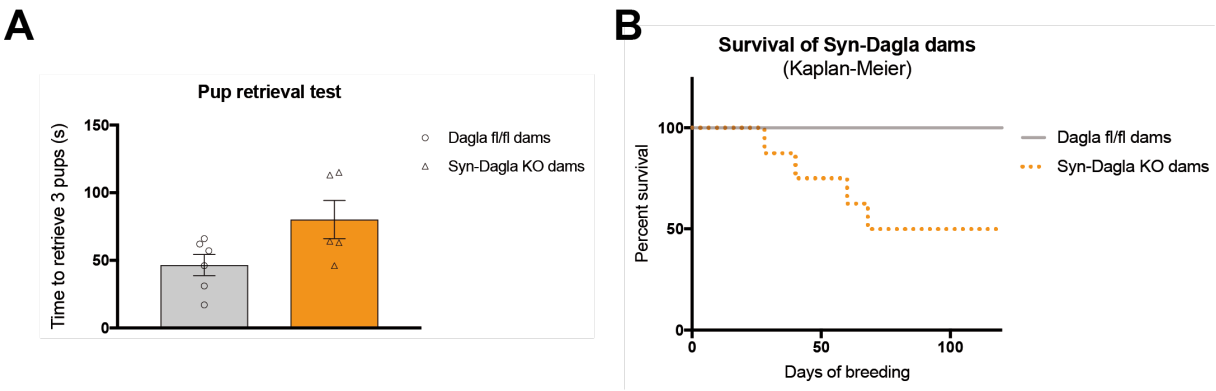


Figure 3.13 Maternal care behavior of Syn-Dagla KO dams

(A) *Syn-Dagla KO dams did not show any differences in the pup retrieval test. All Syn-Dagla KO dams retrieved three of their pups within two minutes similar to WT controls. Values represent mean ± SEM; n= 6 animals/group.* ***(B)*** *Kaplan-Meier survival analysis of Syn-Dagla KO dams: Syn-Dagla KO dams showed increased mortality during breeding compared to Dagla fl/fl control mice. In the course of four months of breeding, half of Syn-Dagla KO dams died during pregnancy or while giving birth.*

Conspicuously, we observed that during the breeding episode many Syn-Dagla KO dams died from seizures during pregnancy or giving birth (Figure 3.13B), even though survival of Syn-Dagla KO mice was normal in general.

3.1.6 Adult neurogenesis in Syn-Dagla mice

Adult neurogenesis, the formation of new neurons and astrocytes during adulthood, is involved in several memory and learning processes, as well as in the pathology of depression (A. S. Hill, Sahay, and Hen 2015; Ramirez-Amaya et al. 2006). To investigate adult neurogenesis, Syn-Dagla KO and control mice were injected with the thymidine analogue BrdU for three consecutive days. BrdU is incorporated in the DNA of dividing cells and was detected by immunohistochemistry. To investigate proliferation of neural progenitor cells, mice were perfused one day after the last injection and the number of BrdU-positive cells in KO and control mice was counted and compared. For the analysis of survival of progenitor cells, another cohort of mice was killed 21 days after the last BrdU injection and numbers of BrdU-positive cells were compared in KO and control mice. Furthermore, a co-

labelling of BrdU-positive cells with neuronal marker NeuN or astrocytic markers GFAP and S100β were analyzed to identify changes in differentiation of progenitor cells.

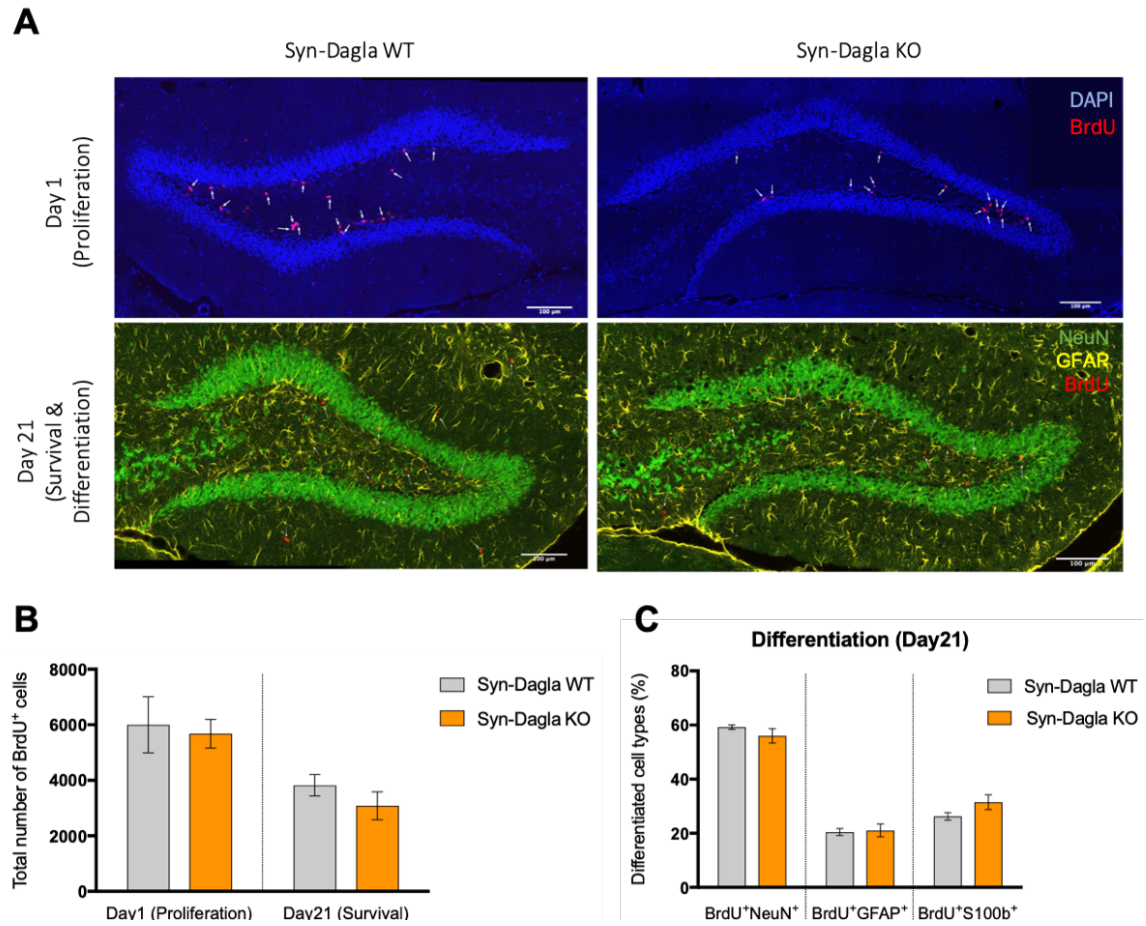


Figure 3.14 Adult hippocampal neurogenesis in Syn-Dagla mice

Syn-Dagla KO mice and control mice were treated with intraperitoneal injections of BrdU (50 mg/kg) for 3 consecutive days. 24 h after the last BrdU injection the proliferation of neural stem cells in dentate gyrus was studied. Another group of mice was perfused 21 days after the last BrdU injection to investigate survival and differentiation of neural progenitor cells in the dentate gyrus. ***(A)*** *Representative immunohistochemistry stainings of Syn-Dagla KO and control mice. Syn-Dagla KO mice show similar number of BrdU-positive cells (red) 1 day after the last injection. 21 days after BrdU injections, the number of BrdU positive cells does not differ between Syn-Dagla KO mice and controls. Additionally, brains were stained with an astrocytic marker GFAP (yellow) and a neuronal marker NeuN (green) to investigate differentiation, which was unchanged (scale bar: 100 µm).* ***(B)*** *The number of BrdU-positive cells in dentate gyrus of Syn-Dagla KO mice was similar to controls, one and 21 days after BrdU injections.* ***(C)*** *Differentiation of progenitor cells in dentate gyrus of Syn-Dagla KO mice. BrdU-positive cells were analyzed for co-expression of neuronal marker NeuN and astrocytic markers GFAP and S100β. Values represent mean ± SEM; n = 4 animals/group, 6 analyzed pictures/animal. Students t-test.*

Syn-Dagla KO mice did not show differences in the number of BrdU-positive days one day after the last injection, suggesting normal proliferation of progenitor cells compared to controls. 21 days after BrdU injections a similar number of BrdU-positive cells was detected in Syn-Dagla KO mice and WT controls. Differentiation in Syn-Dagla KO mice was unchanged compared to control mice (Figure 3.14C).

To determine if the transcription of any proteins involved in adult neurogenesis is changed in Syn-Dagla KO mice, we performed a transcription analysis in hippocampus of those mice using quantitative real-time PCR. We found an expected decrease of *Dagla* expression in Syn-Dagla KO mice compared to littermate controls. Furthermore, transcription of brain derived neurotropic factor (BDNF) was increased in Syn-Dagla KO mice.

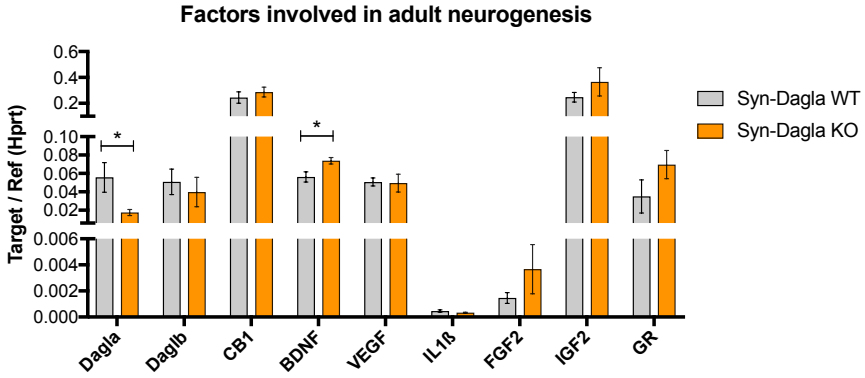


Figure 3.15: Transcriptional analysis of proteins involved in adult neurogenesis and the endocannabinoid system in the hippocampus of Syn-Dagla KO mice.

Dagla transcription was reduced in the hippocampus of Syn-Dagla KO mice compared to controls (p=0.034). Expression of Daglb and CB1 was unchanged. Brain derived neurotropic factor (BDNF) was upregulated in hippocampus of Syn-Dagla KO mice (p=0.006), while vascular and endothelial growth factor (VEGF), interleukin1beta (IL1β), fibroblast growth factor 2 (FGF2), insulin-like growth factor2 (IGF2) and glucocorticoid receptor (GC) expression was not changed in Syn-Dagla KO mice. Values represent mean ± SEM; n=10 animals/group.

In summary, mice lacking DAGLa specifically on neurons, do not show a significant reduction in 2-AG levels, but an increase in DAGLa substrate SAG, as well as a decrease in the 2-AG metabolites AA and prostaglandins. Behaviorally, the deletion of DAGLa on neurons lead to decreased food intake and body weight that was more pronounced in female mice and a decrease in locomotor activity in the home cage. Especially male Syn-Dagla KO mice showed changes in exploratory and anxiety-related behaviors, with an additional increase in corticosterone levels. In depression-related behavioral tests, almost no changes were detected. Adult neurogenesis was unchanged in mice lacking DAGLa on neurons but we observed an upregulation of BDNF expression in the hippocampus of Syn-Dagla KO mice.

3.2 Astrocyte-specific GLAST-CreERT2- and GFAP-Dagla mice

3.2.1 DAGLa expression and endocannabinoid production by astrocytes

To investigate DAGLa in astrocytes, we wanted to use two different conditional mouse lines; GLAST-CreERT2- and GFAP-Dagla KO mice (Figure 3.16A). *Dagla* deletion was first assessed by immunohistochemistry. Whereas GLAST-CreERT2-Dagla KO mice showed a very similar, but weaker DAGLa signal to DAGLa fl/fl controls, there was a marked reduction of DAGLa staining intensity in some brain regions of GFAP-Dagla KO, such as the hippocampus and cortex. Constitutive Dagla KO mice did not show any DAGLa staining (Figure 3.16A). Next, an RT-PCR analysis was performed on whole brain lysates. Again, no significant change in *Dagla* mRNA was found in GLAST-CreERT2-Dagla mice, whilst GFAP-Dagla KO mice showed a significant reduction of *Dagla* mRNA levels (Figure 3.16C). *Dagla* mRNA transcripts were not detected in constitutive Dagla KO brain (Figure 3.16C). These results indicate that astrocytic DAGLa only contributes a relatively small percentage to the overall DAGLa expression in the brain. They also suggest that the deletion of *Dagla* in GFAP-Dagla KO mice is not restricted to astrocytes in some brain regions.

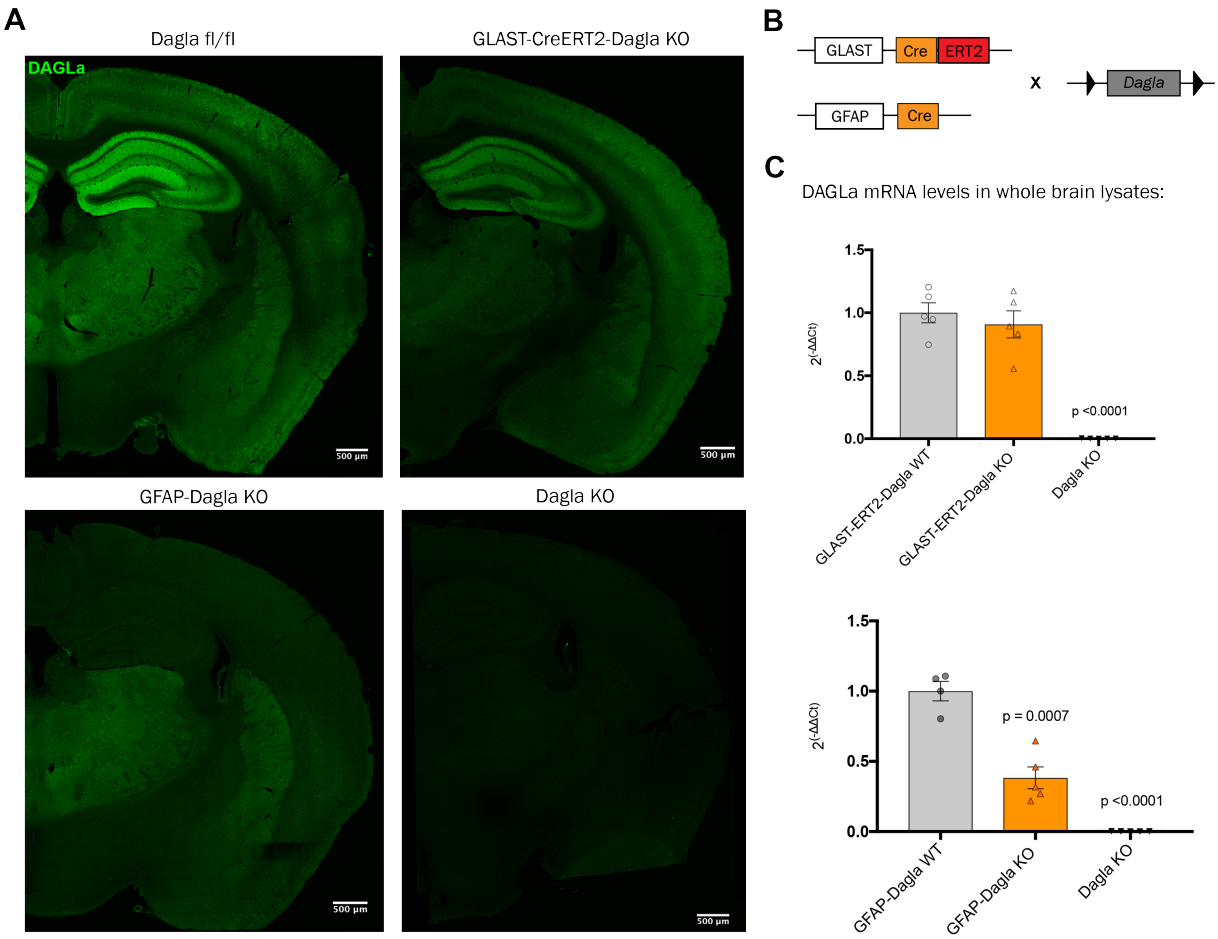


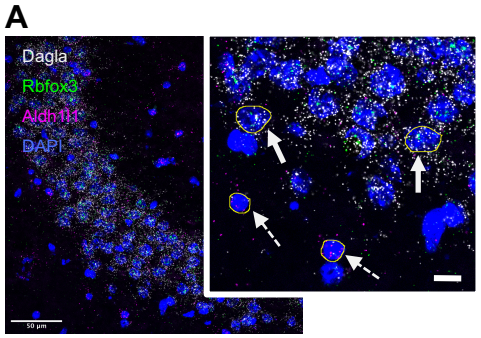
Figure 3.16: Validation of GFAP-Dagla and GLAST-ERT2-Dagla KO efficacy by immunohistochemistry and RT-PCR

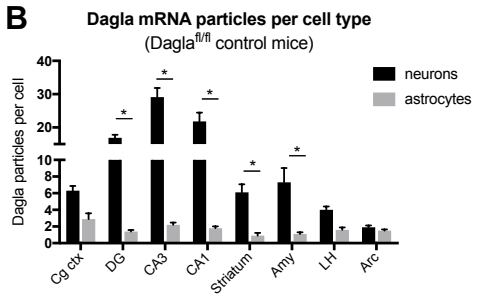
(A) *Representative images of DAGLa (green) immunostainings from brains of Dagla fl/fl control mice, tamoxifen-induced GLAST-CreERT2-Dagla KO, GFAP-Dagla KO and constitutive Dagla KO control mice. DAGLa staining is completely absent in constitutive Dagla KO brains and almost absent in the hippocampus of GFAP-Dagla KO mice. Scale bar: 500 µm.* ***(B)*** *Genetic description of GLAST-CreERT2- and GFAP-Dagla KO mice.* ***(C)*** *Dagla mRNA levels in whole brain lysates normalized to Gapdh. RT-PCR results show no significant reduction of Dagla mRNA expression in GLAST-CreERT2-Dagla KO brain and a 66% reduction of Dagla mRNA expression in GFAP-Dagla KO brain compared to controls.*

The RNAscope method was used to investigate *Dagla* mRNA expression with a cellular resolution (Figure 3.17). The cellular origin of *Dagla* expression was determined through a co-labeling with neuronal (*Rbfox3*) and pan-astrocytic (*Aldh1l1*) markers. In DAGLa fl/fl control mice, the number of *Dagla* particles was up to ten times higher in neurons than in astrocytes (Figure 3.17B). Furthermore, we found that 50-100% of all neurons in control mice expressed *Dagla* transcripts, depending on the brain region, whereas only 40-75% of all astrocytes expressed *Dagla* (Figures 3.17C,D). Together our results indicate that *Dagla* expression in astrocytes accounts for less than 10% of the *Dagla* expression in the brain.

GLAST-CreERT2-Dagla KO mice showed a significant reduction of Dagla transcript in astrocytes in most brain regions analyzed (by *in situ* hybridization). Exceptions were the dentate gyrus and the

striatum where *Dagla* transcripts were still present in ca. 60% and 50%, respectively, of all astrocytes counted. The most pronounced deletion was observed in the cingulate cortex (51%), temporal cortex (38%) and the lateral hypothalamus (36%). A reduction of *Dagla* was also observed in CA1 (32%) and CA3 (21%) regions of hippocampus, as well as in the arcuate nucleus of hypothalamus (27%). Importantly, we did not observe any reduction of *Dagla* in neurons (Figure 3.17C).





C

GLAST-CreERT2-Dagla

Area	Bregma (mm)	% DAGLa positive neurons			% DAGLa positive astrocytes		
		% reduction of Dagla+ cells in KO	WT	KO	% reduction of Dagla+ cells in KO	WT	KO
Cingulate ctx	0.26	1.2	97.6 ± 1.5	96.4 ± 2.4	51.1	53.6 ± 6	26.2 ± 3.5
DG	-1.7	≠	100 ± 0	100 ± 0	-2.3	60.8 ± 4.6	62.2 ± 6.1
CA3	-1.7	≠	100 ± 0	100 ± 0	20.7	73 ± 5.2	57.9 ± 6.5
CA1	-1.7	≠	96.4 ± 1.9	97.6 ± 1.5	31.7	67.9 ± 4.1	46.4 ± 3.1
Striatum	0.26	1.5	88.8 ± 13.6	87.5 ± 22.8	-11.2	50 ± 17.5	55.6 ± 13.6
Temp ctx / Amy	-1.7	5	90.5 ± 5.3	86 ± 8.6	38.1	58.6 ± 5.8	36.3 ± 8.7
Lateral hypoth.	-1.7	10	77.8 ± 8.1	70 ± 11.8	35.8	46.7 ± 21.1	30 ± 17.3
Arc nucleus	-1.7	-4.1	48.8 ± 5.9	50.8 ± 12.3	26.8	36.9 ± 3.6	27 ± 9.5

D

GFAP-Dagla

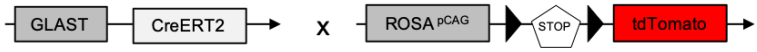
Area	Bregma (mm)	% DAGLa positive neurons			% DAGLa positive astrocytes		
		% reduction of Dagla+ cells in KO	WT	KO	% reduction of Dagla+ cells in KO	WT	KO
Cingulate ctx	0.26	84.3	97.2 ± 6.8	15.3 ± 12.3	93.6	65.3 ± 22	4.2 ± 7
DG	-1.7	77.9	98.8 ± 3.1	21.8 ± 28.2	80.4	64.3 ± 13.3	12.6 ± 10.9
CA3	-1.7	77.8	90.8 ± 3.2	20.2 ± 15.7	78.8	61.9 ± 14.3	13.1 ± 12.6
CA1	-1.7	73	98.8 ± 3.1	17.7 ± 24.2	92.1	65.5 ± 24.3	5.2 ± 7.6
Striatum	0.26	-8	76.7 ± 14.5	82.8 ± 10.4	76.7	46.8 ± 15.7	10.9 ± 6.5
Temp ctx / Amy	-1.7	12.5	92.9 ± 10.1	81.3 ± 22.6	69.2	65.5 ± 10	20.2 ± 13.3
Lateral hypoth.	-1.7	12.3	73.7 ± 14.5	82.8 ± 10.4	76.8	46.9 ± 15.7	10.9 ± 6.5
Arc nucleus	-1.7	0.9	66.2 ± 20.8	65.6 ± 22	61.8	53.2 ± 18	20.2 ± 12.7

Figure 3.17: Dagla expression analysis by RNAscope in situ hybridization.

(A) *A representative image (scale bar 50 µm) of an RNAscope in situ hybridisation assay detecting transcripts of diacylglycerol lipase alpha (Dagla), Rbfox3 (neuronal marker) and Aldh1l1 (astrocytic marker). The magnified inset (scale bar 10 µm) shows examples of encircled cells identified as neurons (bold arrows) or astrocytes (dotted arrows).* ***(B)*** *Dagla mRNA particles in neurons and astrocytes of WT control mice (Dagla fl/fl) in different brain regions: Cingulate cortex (Cg ctx), dentate gyrus (DG), cornu ammonis 3,1 (CA3,1), striatum, amygdala (Amy), lateral hypothalamus (LH) and arcuate nucleus (Arc). RNAscope analysis shows higher Dagla mRNA expression in neurons compared to astrocytes in most brain regions.* ***(C,D)*** *Quantification of Dagla KO efficiency in neurons and astrocytes in different brain regions of GLAST-CreERT2-Dagla* ***(C)*** *and GFAP-Dagla KO* ***(D)*** *mice in comparison to WT littermates (Mean ± SEM, n=3-4 per group). RNAscope analysis was performed by Dr. Este Leidmaa, University of Bonn.*

The specificity of the astrocytic expression of Cre in GLAST-CreERT2 mice was confirmed with the use of RosaTomato reporter mouse strain (Figure 3.18). The reporter gene expression was induced in up to 80% of all astrocytes, whereas only few neurons were tdTomato positive. GFAP-Dagla KO mice showed a strong reduction of *Dagla* transcription in astrocytes of all brain regions investigated. However, these mice also displayed a deletion of *Dagla* in neurons of the hippocampus and the cingulate cortex, thus indicating an off-target effect of the GFAP promoter in some brain regions (Figure 3.17D).

A



B

GLAST-CreERT2-Dagla

Area	% tomato expressor S100ß-positive astrocyte	% tomato expressor NeuN-positive neuron
Cingulate cortex	53.17 ± 13.34	0.73 ± 1.78
DG	63.04 ± 21.36	4.82 ± 4.94
CA3	70.54 ± 14.61	3.18 ± 5.21
CA1	76.68 ± 13.03	1.28 ± 1.78
Striatum	37.02 ± 17.02	0.30 ± 0.76
Amygdala	69.70 ± 7.69	0.13 ± 0.43
Lateral hypothal.	56.02 ± 23.14	0.88 ± 1.16
Arc. Nucleus	79.64 ± 12.77	0.56 ± 1.32

C

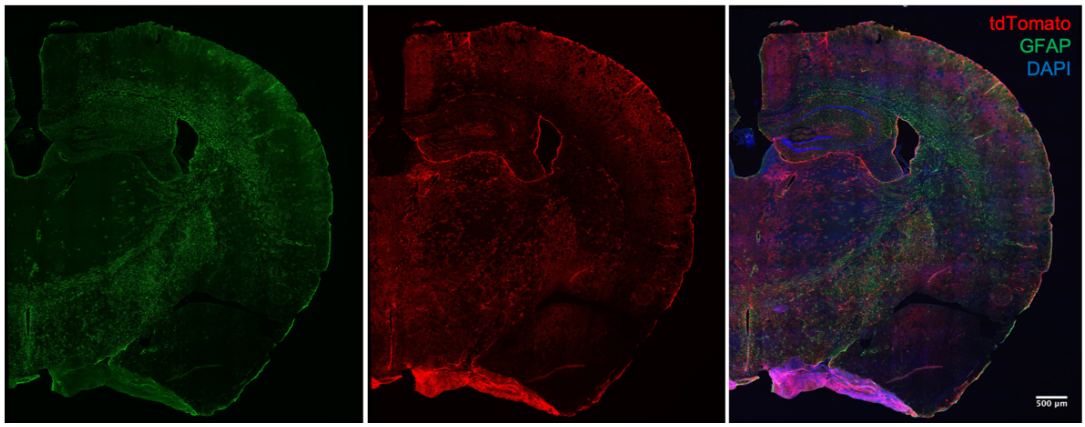


Figure 3.18: Validation of Cre expression in GLAST-ERT2-Dagla KO mice by Rosa-Tomato reporter line.

(A) Genetic description of mice used for validation of Cre expression. (B) Quantification of Cre expression efficiency in astrocytes (% of dTomato cells co-expressing S100beta from all S100beta-positive cells) and cell-type specificity (% of cells co-expressing dTomato and neuronal marker NeuN from all Cre-expressing cells) in different brain regions of GLAST-CreERT2-RosaTomato mice (C) Representative immunohistochemistry picture of a GLAST-CreERT-RosaTomato mouse with green indicating astrocytic-marker GFAP and red representing tdTomato (Scale bar: 500 µm; n=3 per group, 4 of sections per animal for quantification). Rosa-Tomato- IHC and analysis (Table) was performed by PD Dr. Andras Bilkei-Gorzo and Janis Transfeld, University of Bonn.

Next, the levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG), its precursor 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), as well as arachidonic acid (AA), the metabolite of 2-AG, were determined in different brain regions (Figure 3.19). Female, but not male GLAST-CreERT2-Dagla KO mice had a slight but significant decrease in 2-AG and its metabolite arachidonic acid levels (main genotype effect). However, when corrected for multiple comparisons, GLAST-CreERT2-Dagla KO mice did not show significant differences in any of these compounds ($p > 0.05$ in post-hoc tests) (Figure 3.19A-C), which is in good agreement with the more prominent role of neuronal DAGLa to the production of 2-AG. In contrast, GFAP-Dagla KO mice showed significantly decreased 2-AG levels in PFC as well as in hippocampus and hypothalamus, thus supporting the notion of a region-specific off-target effect in this mouse strain (Figure 3.19E). Levels of the DAGLa substrate SAG were significantly increased in PFC, striatum and hippocampus of male GFAP-Dagla KO mice and

in PFC and hippocampus of female GFAP-DAGLa KO mice. Also, the hydrolytic metabolite of 2-AG, AA was decreased in PFC and hippocampus of male, and PFC, striatum and hippocampus of female GFAP-Dagla KO mice (Figure 3.19F).

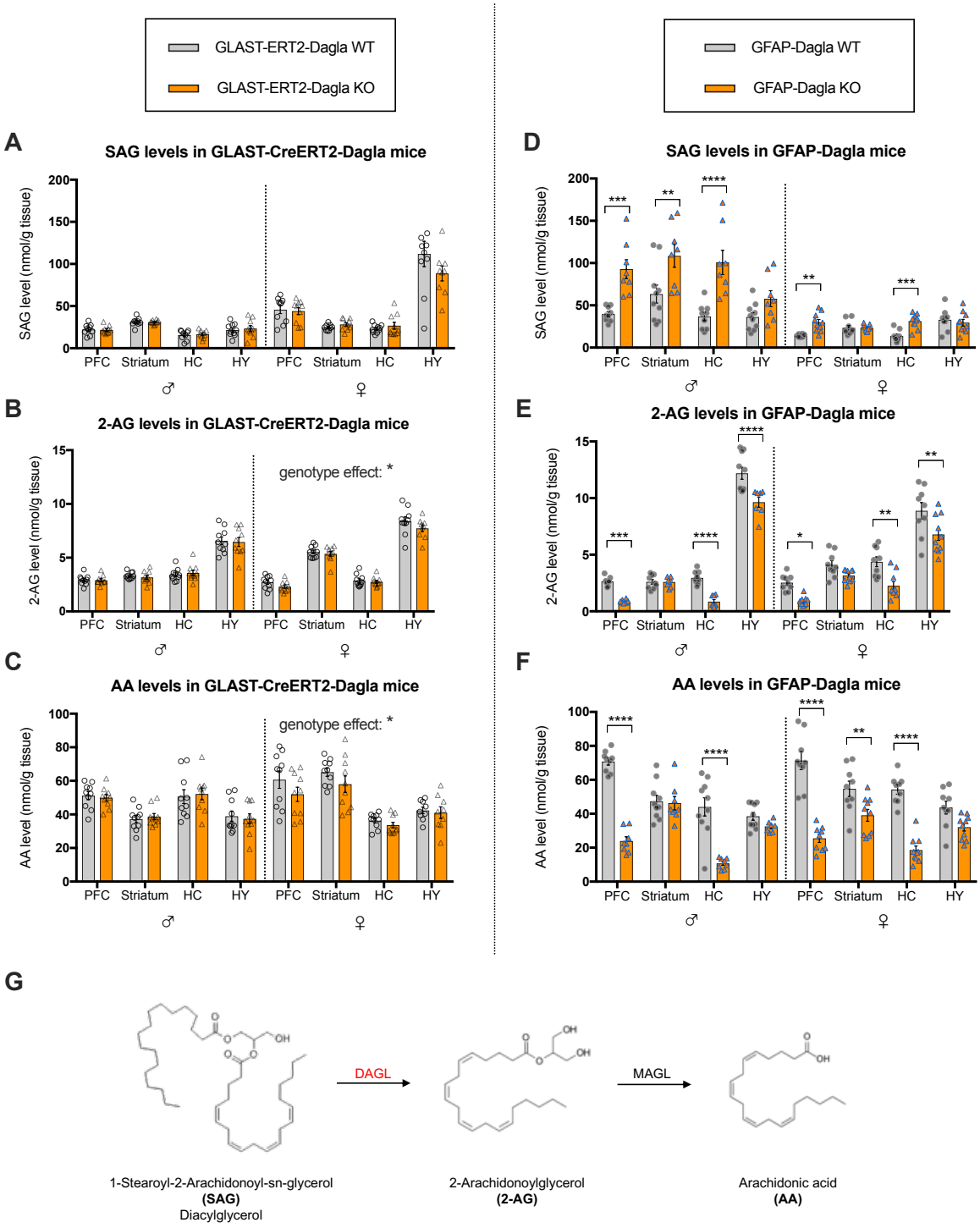


Figure 3.19: Measurements of components of the endocannabinoid system by LC-MS/MS.

(A) *The substrate of DAGLa, 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), was not significantly changed in any of the tested brain regions (prefrontal cortex (PFC), striatum, hippocampus (HC) and hypothalamus (HY) in male or female GLAST-CreERT2-Dagla mice.* ***(B)*** *Female, but not male GLAST-CreERT2-Dagla KO mice showed a significant reduction in 2-arachidonoyl glycerol (2-AG) levels (females: genotype effect: $F_{1,71}$=4.19, p=0.0444; brain region effect: $F_{3,71}$=243.8, p<0.0001). Post hoc analysis was not significant for any of the tested brain regions.* ***(C)*** *Female, but not male GLAST-CreERT2-Dagla mice showed an overall genotype effect in arachidonic acid (AA) levels (females: genotype effect: $F_{1,70}$=4.069, p=0.0475; brain region effect: $F_{3,70}$=25.84, p<0.0001), but no area specific differences between genotypes in the post hoc tests.* ***(D)*** *Male GFAP-Dagla KO mice showed significantly increased levels of SAG in the PFC, striatum and hippocampus, but not in the hypothalamus (genotype effect: $F_{1,62}$=46.88, p<0.0001; brain region effect: $F_{3,62}$=5.813, p=0.0014). Female GFAP-Dagla KO mice showed reductions in SAG levels only in the PFC and hippocampus (genotype effect: $F_{1,66}$=12.62, p=0.0007; brain region effect: $F_{3,66}$=4.253, p=0.0083).* ***(E)*** *Male and female GFAP-Dagla KO mice showed a significant reduction of 2-AG levels in prefrontal PFC, hippocampus and hypothalamus, but not in striatum (males: genotype effect: $F_{1,60}$=60.36,*

p<0.0001; brain region effect: $F_{3,60}$=452.6, p<0.0001; females: genotype effect: $F_{1,67}$=34.06, p<0.0001; brain region effect: $F_{3,67}$=81.08, p<0.0001). ***(F)*** *AA was decreased in the PFC and hippocampus of male GFAP-Dagla KO mice, but not significantly changed in striatum and hypothalamus (genotype effect: $F_{1,62}$=87.91, p<0.0001; brain region effect: $F_{3,62}$=17.26, p<0.0001; interaction: $F_{3,62}$=21.84, p<0.0001). In female GFAP-Dagla KO mice, AA was lower in all tested brain regions, except for hypothalamus (genotype effect: $F_{1,67}$=123.4, p<0.0001; brain region effect: $F_{3,67}$=6.185, p=0.0009; interaction: $F_{3,67}$=10.99, p<0.0001)* ***(G)*** *Schematic representation of the synthesis and metabolism of 2-AG by the enzymes DAGL and monoacylglycerol lipase (MAGL). Values represent mean ± SEM; n (GLAST) = 10 animals/group; n (GFAP) = 8-10 animals/group. Measurements were performed by Sandra Glasmacher, University of Bern.*

Prostaglandin E2 levels were significantly decreased in the hypothalamus of male GLAST-CreERT2-Dagla KO mice compared to their littermate controls (Figure 3.20A). Otherwise we did not observe any changes in prostaglandins in GLAST-CreERT2-Dagla KO mice (Figure 3.20B). In male, as well as in female GFAP-Dagla KO mice, prostaglandin E2 levels were significantly reduced in PFC and hippocampus compared to littermate controls (Figure 3.20C). Prostaglandin D2 levels showed a significant reduction in PFC, striatum and hippocampus of male GFAP-Dagla KO mice. In female mice, prostaglandin D2 levels were decreased in PFC and hippocampus in comparison to control mice (Figure 3.20D).

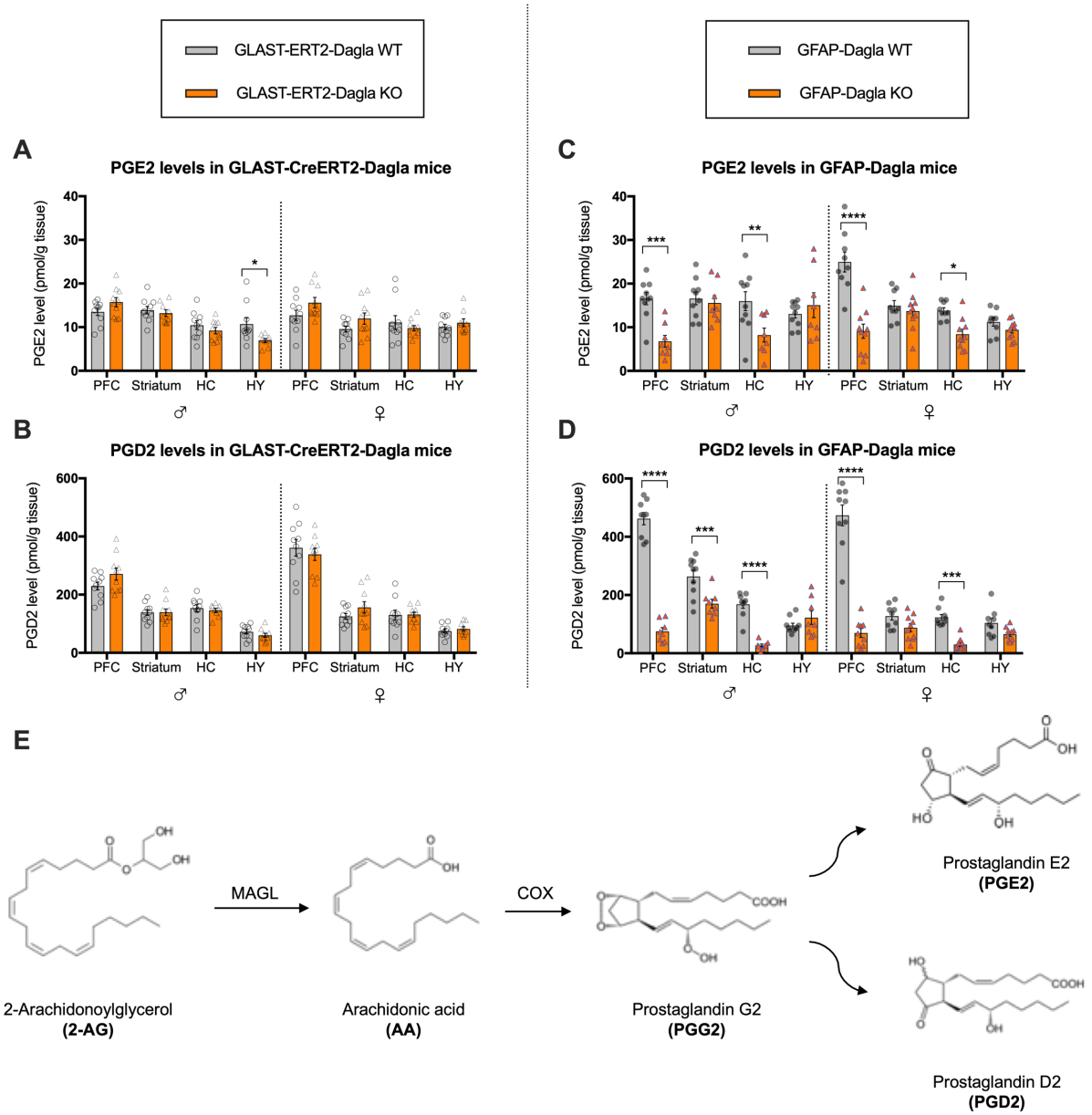


Figure 3.20 Measurements of prostaglandins.

(A) Prostaglandin-E2 (PGE2), was significant decreased in hypothalamus of male GLAST-CreERT2-Dagla KO mice compared to control mice. In other brain regions there were no differences in PGE2 levels neither in male nor in female GLAST-CreERT2-Dagla KO mice. (B) Prostaglandin-D2 (PGD2) levels did not differ in male or female GLAST-CreERT2-Dagla mice in any of the tested brain regions. (C) PGE2 levels were significantly decreased in PFC and hippocampus of male and female GFAP-Dagla KO mice compared to their controls (males: genotype effect: $F_{1,64}$=11.3, p=0.0013; interaction: $F_{3,64}$=5.161, p=0.0029; females: genotype effect: $F_{1,68}$=42.05, p<0.0001; brain region effect: $F_{3,68}$=10.8, p<0.0001; interaction: $F_{3,68}$=13.09, p<0.0001).(D) Male GFAP-Dagla KO mice showed significant decrease of PGD2 levels in PFC, striatum and hippocampus but not in hypothalamus (genotype effect: $F_{1,62}$=163.6,p<0.0001; brain region effect: $F_{3,62}$=51.07,p<0.0001; interaction: $F_{3,62}$=55.53,p<0.0001). Female GFAP-Dagla KO mice showed significant reductions in PGE2 levels in PFC and hippocampus compared to WT controls (genotype effect: $F_{1,66}$=150.5, p<0.0001; brain region effect: $F_{3,66}$=59.78, p<0.0001; interaction: $F_{3,66}$=53.73, p<0.0001). (E) Schematic representation of the synthesis of prostaglandins E2 and D2 from 2-AG. Values represent mean ± SEM; n (GLAST) = 10 animals/group; n (GFAP) = 8-10 animals/group. Measurements were performed by Sandra Glasmacher, University of Bern.

As observed previously in constitutive Dagla KO mice (Y. Gao et al. 2010; Jenniches et al. 2016b; Schurman et al. 2019), the levels of the endocannabinoid anandamide (AEA) were also reduced in

hippocampus of male GFAP-Dagla KO mice and in striatum and hippocampus of female GFAP-Dagla KO mice (Figure 3.21B). GLAST-CreERT2-Dagla KO mice did not show any changes in anandamide levels.

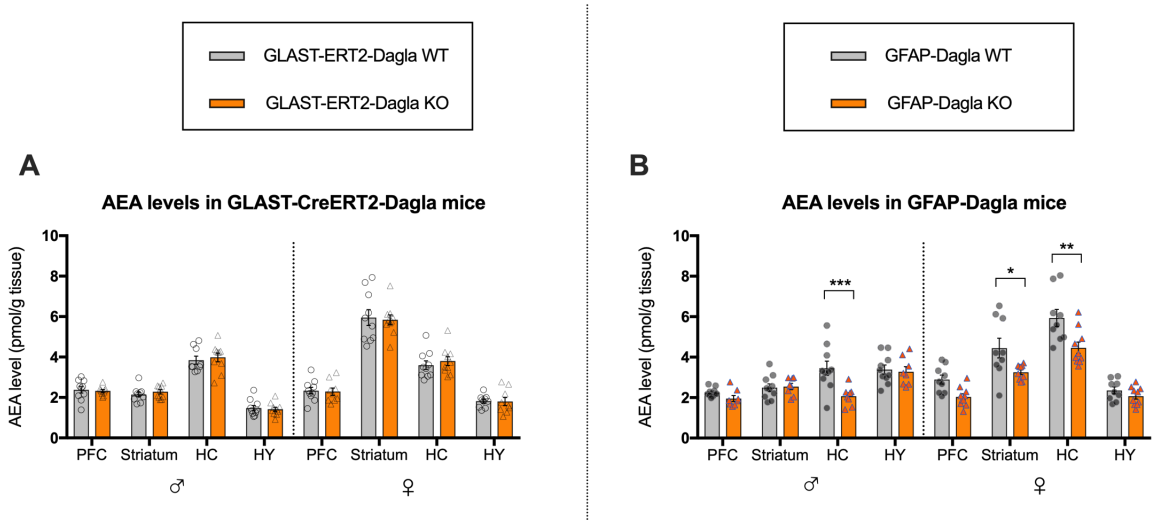


Figure 3.21 Measurements of anandamide (AEA).

(A) *Levels of another endogenous cannabinoid anandamide (AEA) did not differ in male or female GLAST-CreERT2-Dagla KO mice in any of the tested brain regions.* ***(B)*** *Anandamide levels were significantly reduced in the hippocampus of male GFAP-Dagla KO mice, while there were no changes in PFC, striatum or hypothalamus (genotype effect: $F_{1,64}$=7.946, p=0.006; brain region effect: $F_{3,64}$=11.16, p<0.0001; interaction: $F_{3.64}$=4.459, p=0.007). In female GFAP-Dagla KO mice AEA levels were significantly decreased in striatum and hippocampus, but not in PFC and hypothalamus (genotype effect: $F_{1,68}$=24.57, p<0.0001, brain region effect: $F_{3,68}$=51.7, p<0.0001). Values represent mean ± SEM; n (GLAST) = 10 animals/group; n (GFAP) = 8-10 animals/group. Measurements were performed by Sandra Glasmacher, University of Bern.*

Female GLAST-CreERT2-Dagla KO mice, as well as male GFAP-Dagla KO mice showed a significant genotype effect for slightly reduced corticosterone (CORT) levels, but overall CORT levels were in similar range in all mice and different brain regions (Figure 3.22).

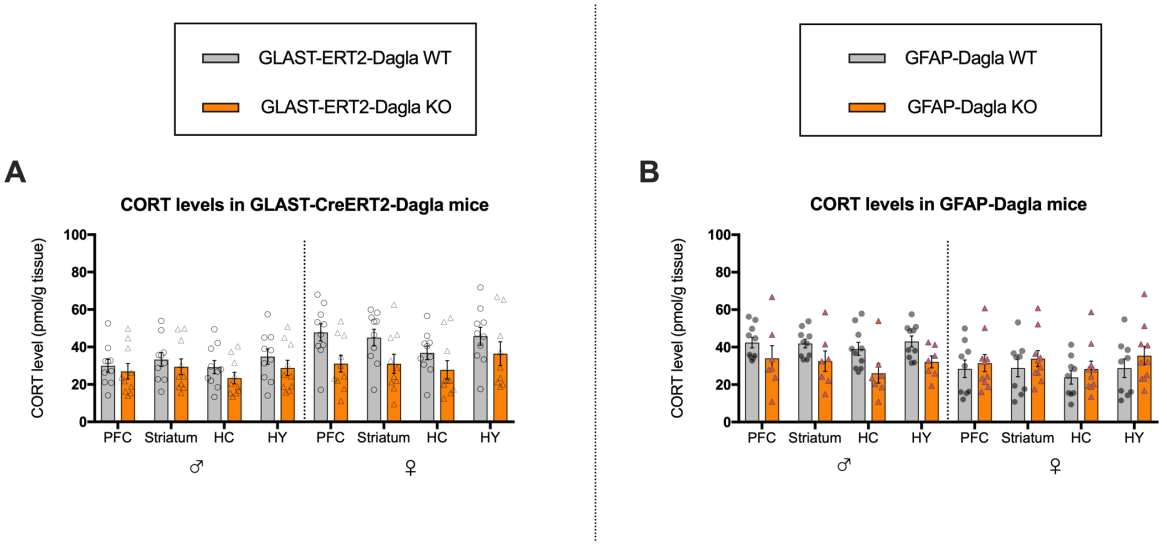


Figure 3.22 Measurements of corticosterone (CORT).

***(A)** Corticosterone (CORT) levels were not changed in any of the tested brain regions between GLAST-CreERT2-Dagla KO mice and control mice. Female, but not male GLAST-CreERT2-Dagla mice showed an overall genotype effect with a slight decrease in KO mice (genotype effect: $F_{1,71}$=13.07, p=0.0006). **(B)** Levels of corticosterone (CORT) did not show significant differences in male or female GFAP-Dagla mice in any of the tested brain regions in the post-hoc tests. Male, but not female GFAP-Dagla mice showed an overall genotype effect in CORT levels, that were slightly decreased in KO mice (genotype effect: $F_{1,60}$=13.83, p=0.0004). Measurements were performed by Sandra Glasmacher, University of Bern.*

In conclusion, GLAST-CreERT-Dagla KO mice show a very specific deletion of *Dagla* in astrocytes that affects up to 51.1% of all astrocytes depending on the brain region. In contrast, GFAP-Dagla KO mice show a higher efficiency of the *Dagla* KO in up to 93.6% of astrocytes but a lower specificity since the KO also partly affects neurons, especially in cortex and hippocampus. *Dagla* mRNA expression was unchanged in whole brain lysates of GLAST-CreERT-Dagla KO mice, whereas GFAP-Dagla KO mice showed a strong reduction of *Dagla* mRNA transcript of around 66%. We were able to measure a slight decrease in the levels of DAGLa product 2-AG and its metabolite AA in female GLAST-CreERT2-Dagla KO mice that could not be detected in male mice. However, male and female GFAP-Dagla KO mice showed a strong reduction of 2-AG levels and 2-AG metabolites, while DAGLa substrate SAG was upregulated. Furthermore, we also measured a decrease of anandamide levels in some brain regions of GFAP-Dagla KO mice that was not observed in GLAST-CreERT2-Dagla KO mice. The validation of GFAP-Dagla KO mice showed that the GFAP-Cre line is not useful to target astrocytes specifically.

3.2.2 Body weight, food intake and survival of GLAST-CreERT2- and GFAP-Dagla KO mice

Neither GLAST-CreERT2-Dagla KO nor GFAP-Dagla KO mice of either gender displayed significant changes in body weight or food intake (Figure 3.23).

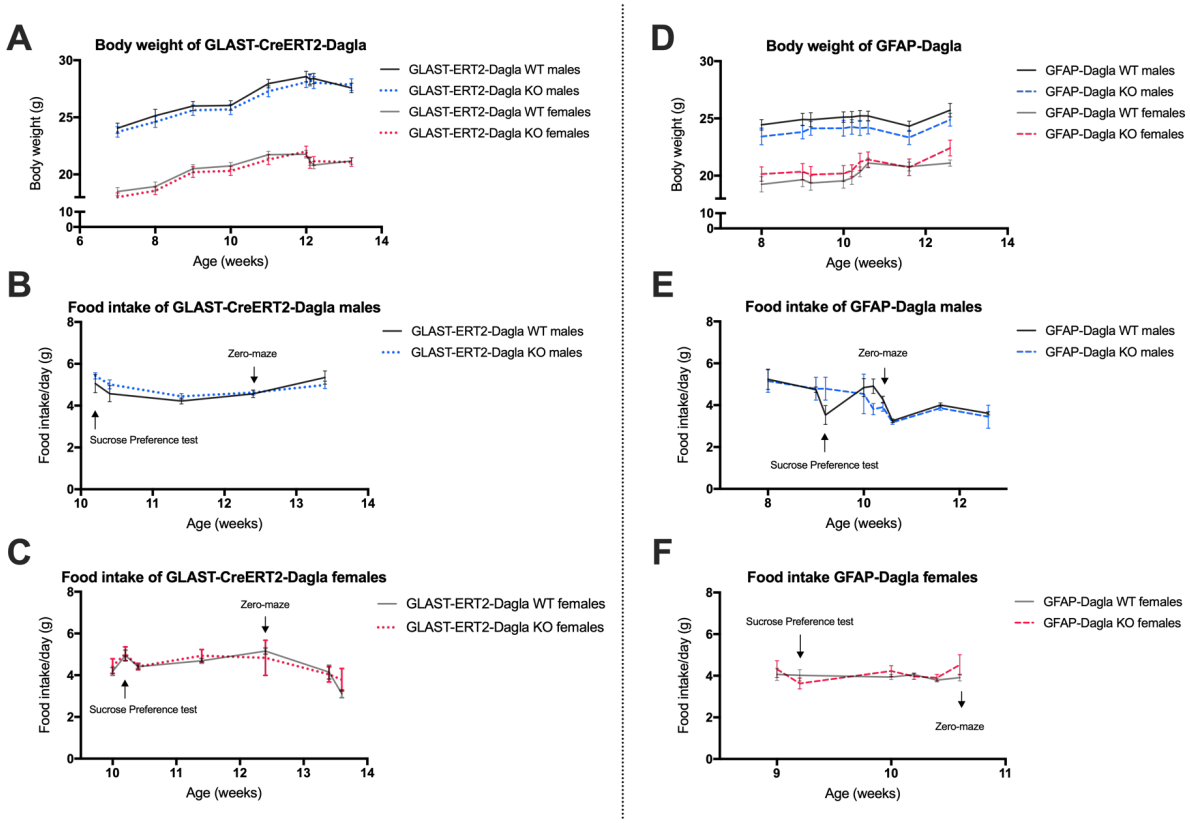


Figure 3.23 Body weight and intake of standard chow in male and female GFAP-Dagla and GLAST-ERT2-Dagla KO mice.

(A) *Body weight of male and female GLAST-CreERT2-Dagla KO mice in comparison to their WT littermate controls (14 animals/group) was not significantly changed (males $F_{1,26}$=0.3207 p=0.5760; females $F_{1,27}$=0.1385, p=0.7127) and food intake per day did also not differ between GLAST-CreERT2-DAGLa KO mice and their WT littermates either in males ($F_{1,26}$=0.5347 p=0.4712)* ***(B)*** *or females ($F_{1,27}$=0.1539 p=0.6981)* ***(C)****.* ***(D)*** *Body weight of GFAP-Dagla KO male mice was slightly decreased compared to WT littermates but did not reach significance (genotype effect: $F_{1,16}$=2.062, p=0.1703; time effect $F_{8,182}$=9.629, p<0.0001). Female GFAP-DAGLa KO mice did not show weight differences compared to littermate controls (10 animals/group; genotype effect: $F_{1,15}$=0.7471, p=0.4010; time effect: $F_{8,120}$=15.8, p<0.0001).* ***(E, F)*** *Food intake per day was not changed in either male (genotype effect: $F_{1,16}$=0.0129, p=0.9107; time effect: $F_{10,160}$=17.91, p<0.0001) or female (genotype effect: $F_{1,14}$=0.5725, p=0.4618; time effect: $F_{5,70}$=1.036, p=0.4036) GFAP-Dagla KO mice. Values represent mean ± SEM.*

Furthermore, male and female GLAST-CreERT2-Dagla KO mice showed normal survival in comparison to littermate controls during the behavioral testing phase. In contrast, we observed decreased survival in GFAP-Dagla KO mice. During the testing phase 20% of male and female GFAP-

Dagla KO mice died, whereas all littermate controls survived (Figure 3.24A). Our observations showed that most of the GFAP-Dagla KO mice seemed to die of epileptic seizures.

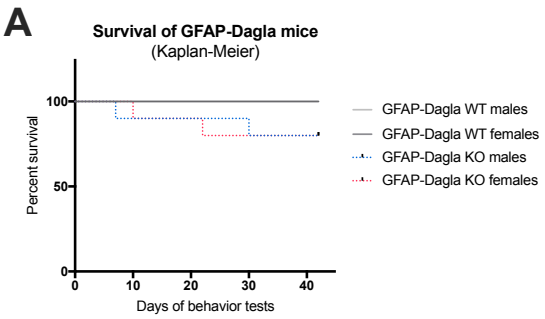


Figure 3.24 Kaplan-Meier survival analysis of male and female GFAP-Dagla KO mice

(A) *GFAP-Dagla KO mice displayed increased mortality (20%) compared to GFAP-Dagla WT. In the male and the female group, 2 of 10 GFAP-Dagla KO mice died from seizures, while none of the control mice died during behavioral testing.*

Since GFAP-Dagla KO mice were suffering from seizures, we were interested if we could see signs of injury in the brains of the mice. Furthermore, we wanted to investigate if the *Dagla* deletion in astrocytes might lead to a change in astrocyte morphology. Therefore, the area covered with astrocytic marker GFAP in the dentate gyrus of astrocyte-specific Dagla KO mice was investigated (Figure 3.25). GFAP-Dagla KO mice showed a significantly increased area covered with GFAP in the dentate gyrus compared to their littermate controls (Figure 3.25B). However, the number of astrocytes measured in a S100β immunostaining was not altered (Figure 3.25D). This result suggests a change in astrocyte morphology in GFAP-Dagla KO mice. In GLAST-CreERT2-Dagla KO mice a slight but not significant increase in area covered with GFAP was observed. Only in the polymorph layer (hilus), the increase was significant (Figure 3.25C).

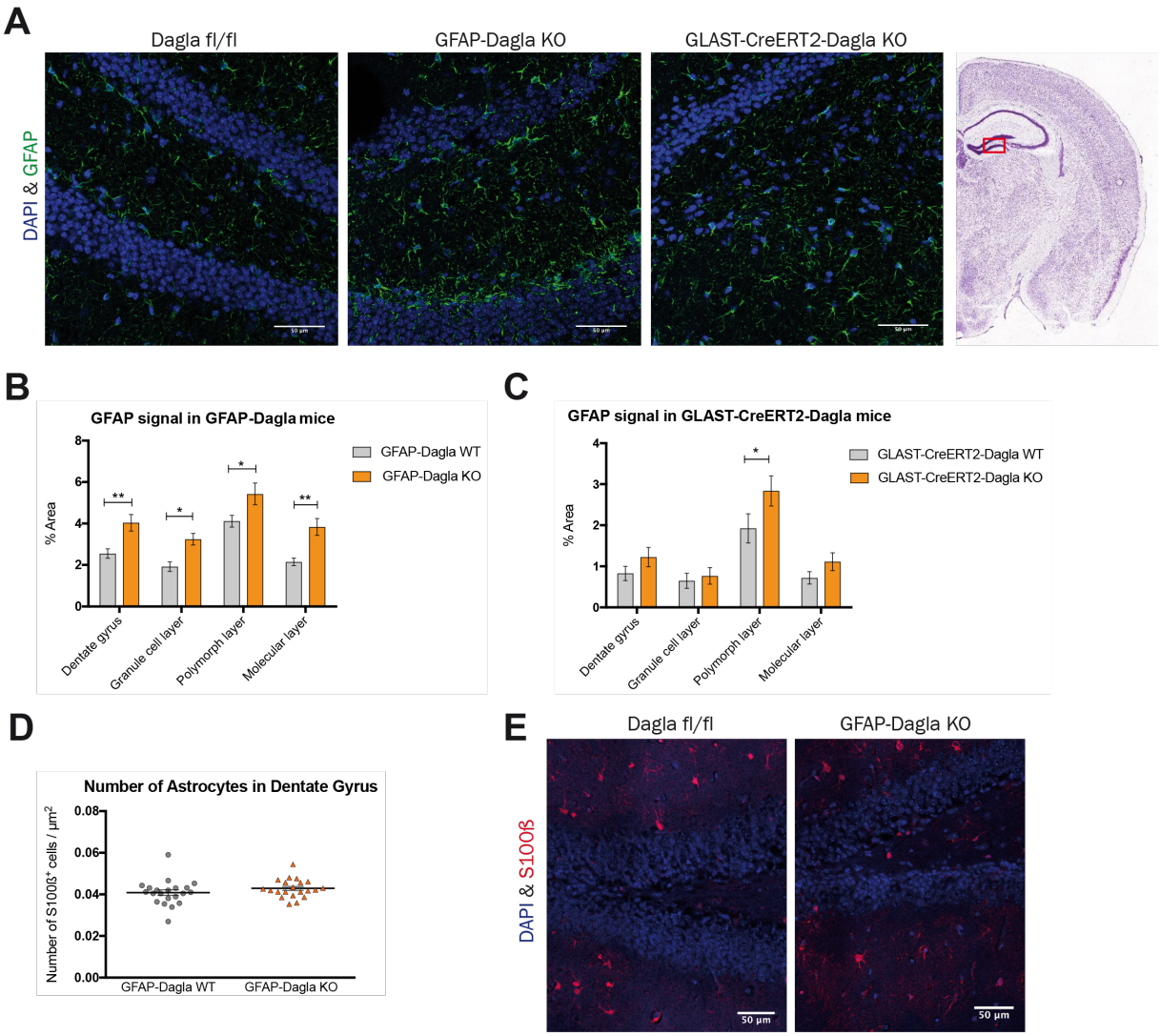


Figure 3.25 Analysis of astrogliosis in dentate gyrus of GFAP-Dagla and GLAST-ERT2-Dagla KO mice

*(A) Representative immunohistochemistry stainings of Dagla fl/fl control brain, GFAP-Dagla KO and GLAST-CreERT2-Dagla KO brain. GFAP-Dagla KO mice showed increased GFAP signal (green) in dentate gyrus compared to control mice whereas GFAP signal in GLAST-CreERT2-Dagla KO mice seemed to be unchanged compared to control mice (blue: DAPI; green: GFAP; scale bar: 50um). (B) Analysis of percentage of area covered with GFAP in dentate gyrus of GFAP-Dagla KO mice compared with controls. In dentate gyrus and each subregion of dentate gyrus, GFAP signal was significantly increased in GFAP-Dagla KO mice. (C) GFAP signal in dentate gyrus of GLAST-CreERT2-Dagla KO mice was not significantly increased compared to controls. Only in polymorph layer of dentate gyrus the GFAP signal was increased compared to controls. (D) Number of S100β-positive cells per µm² in dentate gyrus did not differ between GFAP-Dagla KO mice and WT controls. (E) Representative immunohistochemistry pictures of S100β staining in GFAP-Dagla KO mice and control WT (values represent mean ± SEM; n=4 mice per genotype; 6 analyzed pictures per animal; 2 way-ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001).*

To investigate if the morphological changes in astrocytes also occur in other brain regions that are not involved in neurogenic processes, the experiment was also performed in retrosplenial cortex (RSC) and amygdala. In RSC of GFAP-Dagla KO mice, the area covered with GFAP was significantly increased in comparison to WT controls, whereas GFAP signal in the amygdala was unchanged (Figure 3.26B). In GLAST-CreERT2-Dagla KO mice no changes in GFAP signal in RSC nor amygdala were observed (Figure 3.26C).

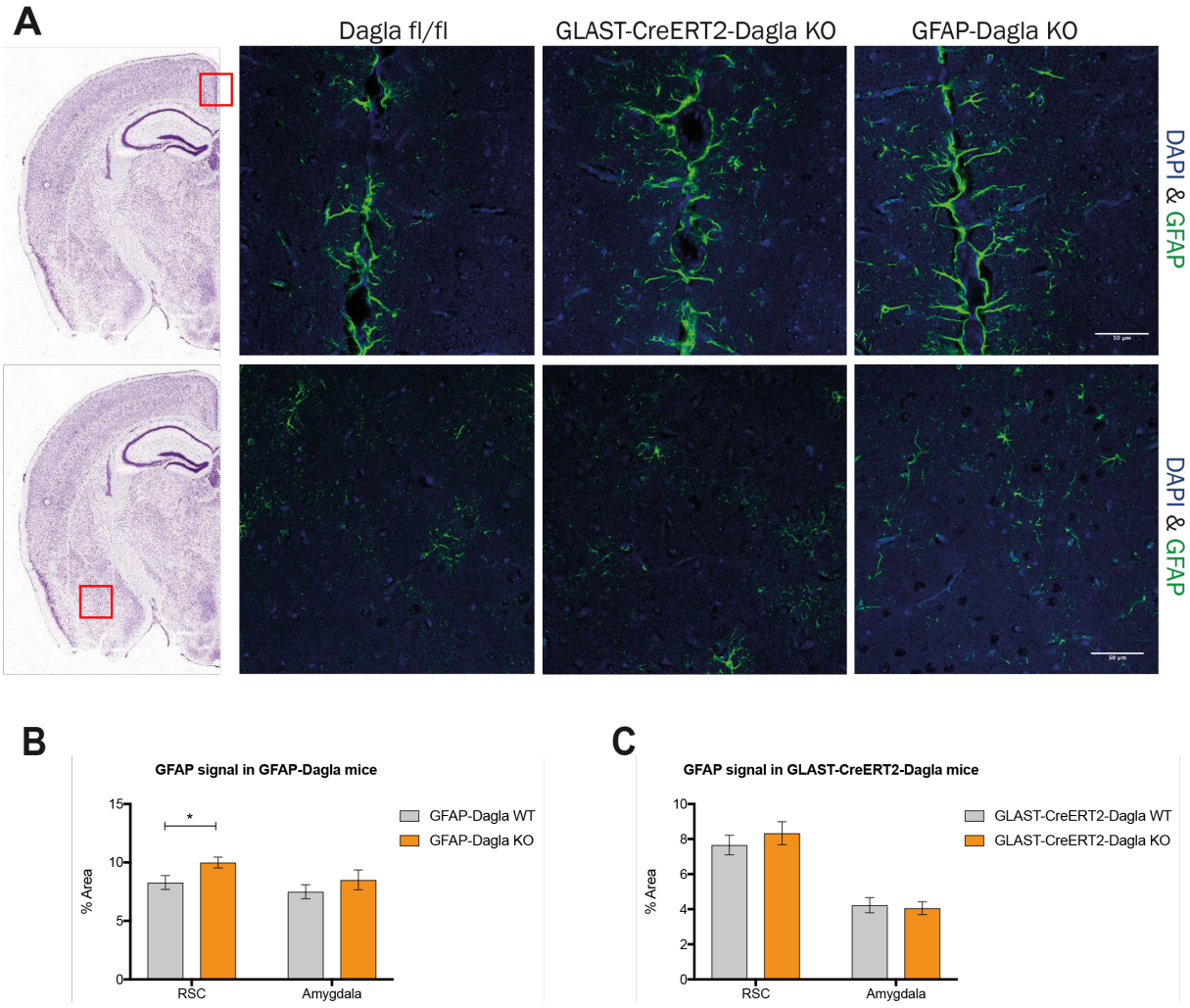


Figure 3.26: Analysis of astrogliosis in retrosplenial cortex (RSC) and amygdala of GFAP- and GLAST-CreERT2-Dagla KO mice.

(A) *Representative immunohistochemistry pictures of retrosplenial cortex (upper row) and amygdala (bottom row). GFAP signal (green) seems to be higher in retrosplenial cortex of GFAP-Dagla KO mice (scale bar: 50 µm). Exemplary images (left) of the plane of analysis from Mouse brain atlas (Franklin and Paxinos, 3rd edition)* ***(B)*** *Quantification of area covered with GFAP signal in retrosplenial cortex and amygdala of GFAP-Dagla KO mice. GFAP covered area is significantly increased in GFAP-Dagla KO mice compared to control mice.* ***(C)*** *Quantification of area covered with GFAP signal in retrosplenial cortex and amygdala of GLAST-CreERT2-Dagla KO mice compared to littermates. There were no changes in GFAP signal in both areas.(Values represent mean ± SEM; n=4 mice per genotype; 6 analyzed pictures per animal; 2 way-ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001.*

In summary, GFAP-Dagla KO mice show increased mortality, probably due to seizures, with an additional change in astrocyte morphology in dentate gyrus and RSC, both areas where the Dagla KO affects also many neurons in addition to astrocytes. In contrast, GLAST-CreERT2-Dagla KO mice show normal survival and only a slight change in astrocyte morphology in the dentate gyrus, whereas RSC and amygdala showed normal astrocyte morphology. Body weight was unaltered in astrocyte-specific GLAST-CreERT2- or GFAP-Dagla KO mice.

3.2.2.1 Tamoxifen-induced hernia in male GLAST-CreERT2-Dagla WT and KO mice

Male and female GLAST-CreERT2-Dagla KO mice were injected with tamoxifen to induce the DAGLa KO in astrocytes of those mice. To create proper controls, WT littermates were injected with tamoxifen as well. Three weeks after the injections, we observed an abdominal swelling only in male GLAST-CreERT2-Dagla KO mice and controls. Female mice did not show this swelling after the injections (Figure 3.27).

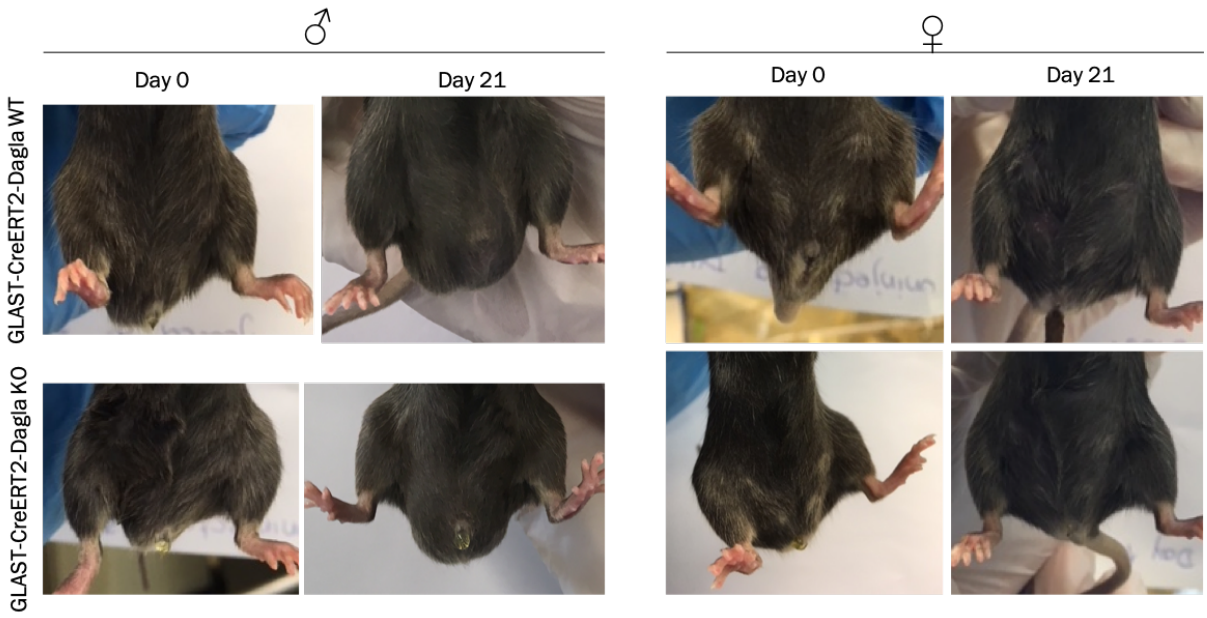


Figure 3.27 Tamoxifen-induced abdominal swelling in male mice

Male, but not female, GLAST-CreERT2-Dagla mice developed hernia after tamoxifen injections. Hernia was independent of the genotype of the mice. Pictures were taken before the first tamoxifen-injection (day 0) and three weeks after tamoxifen-injections (day 21), when behavioral testing was started.

This abdominal swelling after tamoxifen injections in male mice was also observed by Ma *et al*., 2015, who found that tamoxifen activates collagenases which leads to the development of a hernia only in male mice (X. Ma et al. 2015).

3.2.3 Exploratory and anxiety-related behaviors in GLAST-CreERT2- and GFAP-Dagla KO mice

There was no difference in open-field exploratory activity between GLAST-CreERT2-Dagla KO mice and WT controls (Figure 3.28A), as evaluated by the total distance moved and the activity in the central area of the open field. There were also no sex differences in this strain. In contrast, GFAP-

Dagla KO mice showed a significantly increased distance traveled and they spent significantly less time in the center of the box in comparison to WT controls (Figure 3.28B).

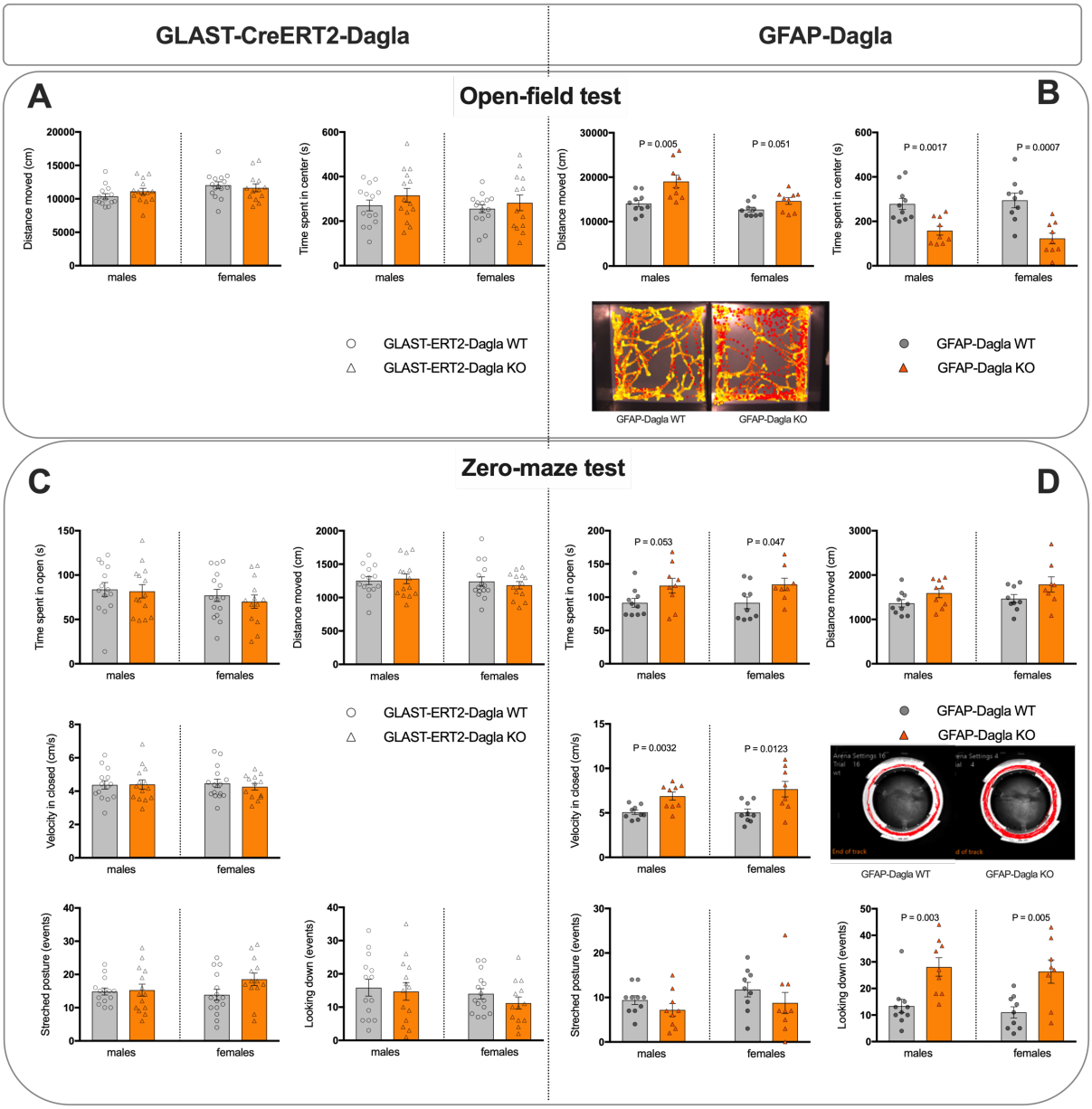


Figure 3.28: Analysis of anxiety-related behavior in astrocyte-specific Dagla KO mice.

(A) *Neither male nor female GLAST-CreERT2-Dagla mice showed changes in the open-field test; in the time spent in center (males: p=0.2646; females: p= 0.4879) or in distance moved (males: p=0.2336; females: p=0.6191) during 30 minutes of testing.* ***(B)*** *GFAP-Dagla KO mice from both genders spent significantly less time in the center of the open-field compared to controls. Additionally, GFAP-Dagla KO males traveled a significantly greater distance in comparison to male GFAP-Dagla WT mice. Although females seemed to show a similar phenotype in the distance moved, this failed to reach significance. Representative tracking maps of GFAP-Dagla KO and WT mice show an overview of movements during 30 minutes in the open-field, with yellow color indicating low and red high velocity. GFAP-Dagla KO mice show in general increased velocity and greater distance moved, especially in the corners of the open-field compared to WT mice.* ***(C)*** *Male and female GLAST-CreERT2-Dagla KO mice did not show any differences in their behavior in the zero-maze test compared to WT controls. Time spent in the open compartment (males: p=0.8469; females: p=0.4958), distance moved (males: p=0.7717; females: p=0.5489), mean velocity in the closed compartment (males: p=0.9421; females: p=0.5448), looking down and stretched posture behaviors were not changed compared to WT controls.* ***(D)*** *In the zero-maze test, female GFAP-Dagla KO mice spent significantly more time in the open compartments of the maze. Velocity in the closed compartments was significantly increased for both genders in GFAP-Dagla KO mice. Male and female GFAP-Dagla KO mice were*

looking down from the maze more often than their littermate controls. Representative tracking maps of GFAP-Dagla WT and KO mice show the route of one individual mouse in the zero-maze during five minutes. Mean ± SEM; n (GLAST) = 14 animals/group; n (GFAP) = 8-10 animals/group.

In the zero-maze GLAST-CreERT2-Dagla KO mice also showed no significant changes in any of the parameters tested, when compared to WT littermates (Figure 3.28C). However, GFAP-Dagla KO mice spent more time in the open compartments than WT controls (Figure 3.28D), they moved faster in the closed compartments, and they showed an increased "looking down behavior" in the open-compartments. The latter has been interpreted as an indicator of reduced anxiety and increased exploratory behavior (Shepherd et al. 1994). To determine, if GFAP-Dagla KO mice are generally hyperactive, we measured their activity also in the home cage (Figure 3.29). Here, we did not find any difference between GFAP-Dagla KO and WT mice, indicating that the increased activity of GFAP-Dagla KO mice in the open-field and zero-maze tests was induced by the novelty of the situation.

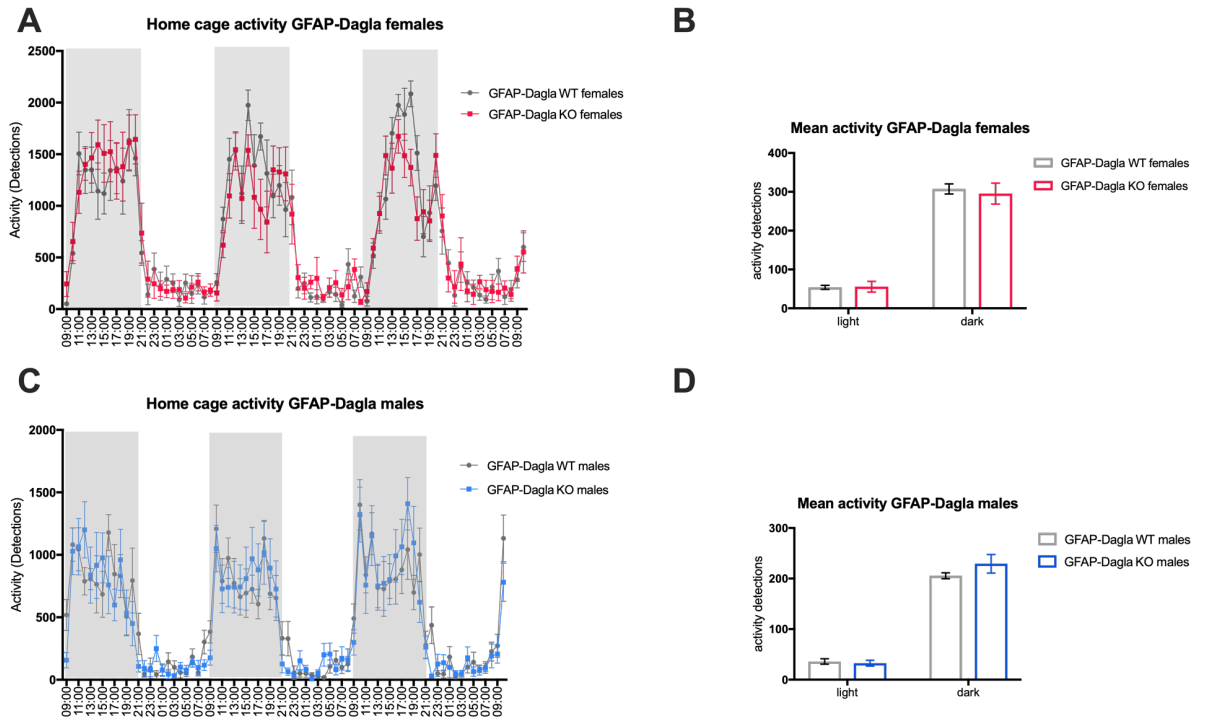


Figure 3.29 Home cage activity measurements of male and female GFAP-Dagla mice.

(A) *Home cage activity measurements of male GFAP-Dagla mice did not show any differences in activity between GFAP-Dagla KO mice and GFAP-Dagla WT control mice in their home cages. GFAP-Dagla KO mice are not hyperactive under normal conditions, without stress (genotype effect: $F_{1,16}$=0.0275, p=0.8703; time effect: $F_{73,1168}$=17,47, p<0.0001).* ***(B)*** *Activity during the light- and dark-phase did also not differ between male GFAP-Dagla KO mice and male controls genotypes (genotype effect: $F_{1,15}$=0.9402, p=0.3476, phase effect: $F_{1,15}$=381.7, p<0.0001.* ***(C)*** *Female GFAP-Dagla KO mice did not show any changes in home cage activity under normal conditions (genotype effect: $F_{1,16}$=0.0832, p=0.7766; time effect: $F_{73,1168}$=23.02, p<0.0001).* ***(D)*** *There were no changes in activity during the light- or the dark-phase between female GFAP-Dagla KO mice and controls (genotype effect: $F_{1,16}$=0.0732, p=0.7903, phase effect: $F_{1,16}$=235.5, p<0.0001). Values represent mean ± SEM; n=8-9 animals/group.*

Taken together, only GFAP-Dagla KO mice with a partial KO of DAGLa also in neurons, showed changes in exploratory and anxiety-related behavior. They spent less time in the center of the open-field box but more time in the open-compartments of the zero-maze. Furthermore, GFAP mice showed increased velocity in the tests, even though their locomotor activity was normal in the home cage.

3.2.4 Depression-like behavior in GLAST-CreERT2- and GFAP-Dagla KO mice

Depressive-like behavior was assessed in the nestlet, sucrose-preference and forced swim tests. In the nestlet test, GLAST-CreERT2-Dagla WT mice shredded almost the entire nestlet in four hours, which was significantly less nestlet shredded for female GLAST-CreERT2-Dagla KO mice (Figure 3.30A). Even after 60 h, the quality of nests of female GLAST-CreERT2-Dagla KO mice was still lower than that of GLAST-CreERT2-Dagla WT mice (Figure 3.31A). This phenotype was even more pronounced in GFAP-Dagla KO mice, which showed a significantly reduced nest building activity during four hours of testing in both sexes (Figure 3.30E). Even after 60 h, only one GFAP-Dagla KO mouse from either sex built a full nest (Figure 3.31B).

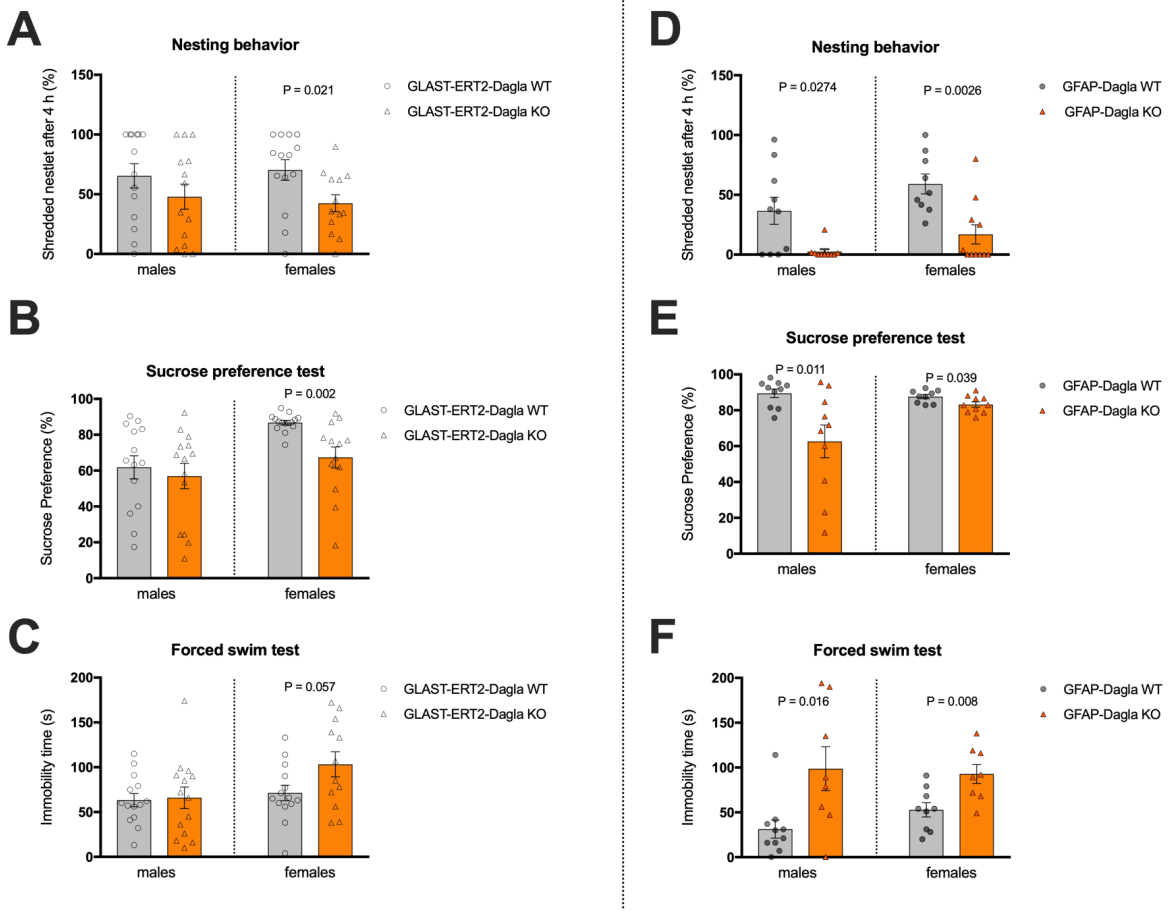


Figure 3.30 Analysis of depression-like behavior in astrocyte-specific Dagla KO mice.

(A) *Nest-building behavior was unchanged in male GLAST-CreERT2-Dagla KO mice compared to WT littermate controls (p=0.2245). Female GLAST-CreERT2-Dagla KO mice show significantly decreased nest building.* ***(B)*** *Anhedonia assessed by the sucrose preference test was not changed in GLAST-CreERT2-Dagla KO males (p=0.6124), whereas female GLAST-CreERT2-Dagla KO mice showed reduced sucrose preference compared to female controls.* ***(C)*** *Female GLAST-CreERT2-Dagla KO mice displayed a tendency for increased immobility in the forced swim test compared to controls, but there was no change in the male group.* ***(D)*** *Male and female GFAP-Dagla KO mice showed significantly decreased nest building compared to their controls.* ***(E)*** *Sucrose preference was reduced in both, male and female GFAP-Dagla KO mice.* ***(F)*** *In forced swim test, GFAP-Dagla KO mice of both genders showed increased immobility time. Mean ± SEM; n (GLAST) = 14 animals/group; n (GFAP) = 8-10 animals/group.*

In the sucrose preference test, GLAST-CreERT2-DAGLa KO females displayed an anhedonia phenotype (Figure 3.30B) that was not observed in males. In GFAP-Dagla KO strain, both sexes showed a significantly reduced sucrose preference (Figure 3.30E).

In the forced swim test, female GLAST-CreERT2-Dagla KO mice seemed to show a higher immobility time compared to their WT controls, but this difference just failed to reach significance (Figure 3.30C; females: p = 0.057). In contrast, male and female GFAP-Dagla KO mice both showed a significantly increased immobility time (Figure 3.30F).

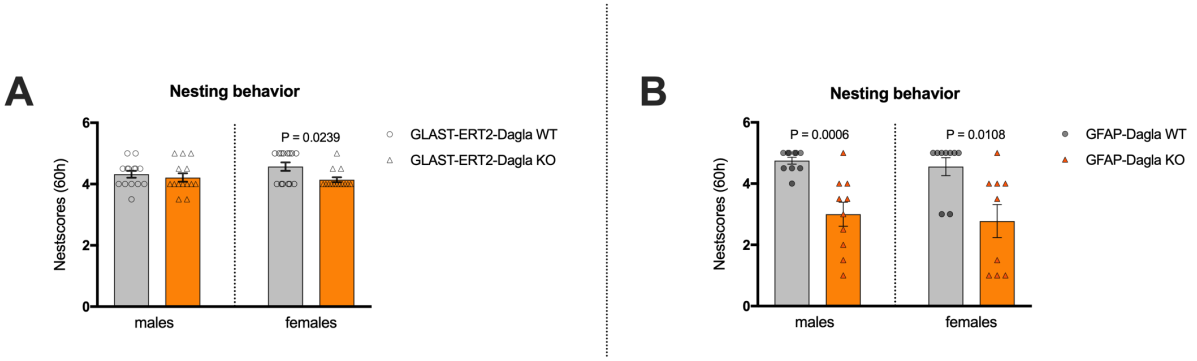


Figure 3.31 Nest scores after 60 h.

Nests of GFAP-Dagla and GLAST-CreERT2-Dagla mice were evaluated 60 h after providing new nestlets according to Deacon et al. 2006. ***(A)*** *Male GLAST-CreERT2-Dagla KO mice achieved almost the same nest scores like their WT controls. Female GLAST-CreERT2-Dagla KO mice did completely shred the nest after 60 h like WT controls; however, the shredded nestlet pieces of most KO mice were distributed all over the cage and not assembled to a fluffy nest like for WT controls (p = 0.0125).* ***(B)*** *Most male and female GFAP-Dagla KO mice did not build nests even after 60 h in contrast to their WT controls. Values represent mean ± SEM; n=9-10 animals/group*

Altogether, these results indicate a depression-related behavioral phenotype in female GLAST-CreERT2-Dagla KO mice lacking *Dagla* expression in astrocytes, which is more pronounced in GFAP-Dagla KO that also have a *Dagla* deletion in some neuronal cells.

3.2.5 Astrocytic DAGLa contributes to maternal care behaviors

The pup retrieval test was performed to investigate maternal care and depressive-like behavior. GLAST-CreERT2-Dagla KO dams either failed to retrieve three of their pups from the opposite corner of the home cage to the nest or they needed significantly more time compared to WT controls (Figure 3.32A). We also tested GLAST-CreERT2-Dagla KO dams in sensory tests in order to determine if the maternal care phenotype was due to an impaired vision or olfaction. To test olfaction, the time a mouse spent with a cotton swab scented with an odor compared to a cotton swab dipped into water was measured. Both, female GLAST-CreERT2-Dagla WT and KO mice spent significantly more time with the scented cotton swab, suggesting that they were able to smell (two-way ANOVA: p = 0.0003; no genotype effect). Please note that previous analysis of constitutive Dagla KO mice showed that hearing is not impaired in mice lacking *Dagla* (Jenniches et al. 2016b). GLAST-CreERT2-Dagla WT and KO mice have perfect vision; all mice received a score of 3 out of 1-3 (Figure 3.32B). It is noteworthy that non-induced GLAST-CreERT-Dagla KO dams retrieved their pups normally (data not shown), suggesting that this phenotype is not mediated by the heterozygous KO of GLAST protein.

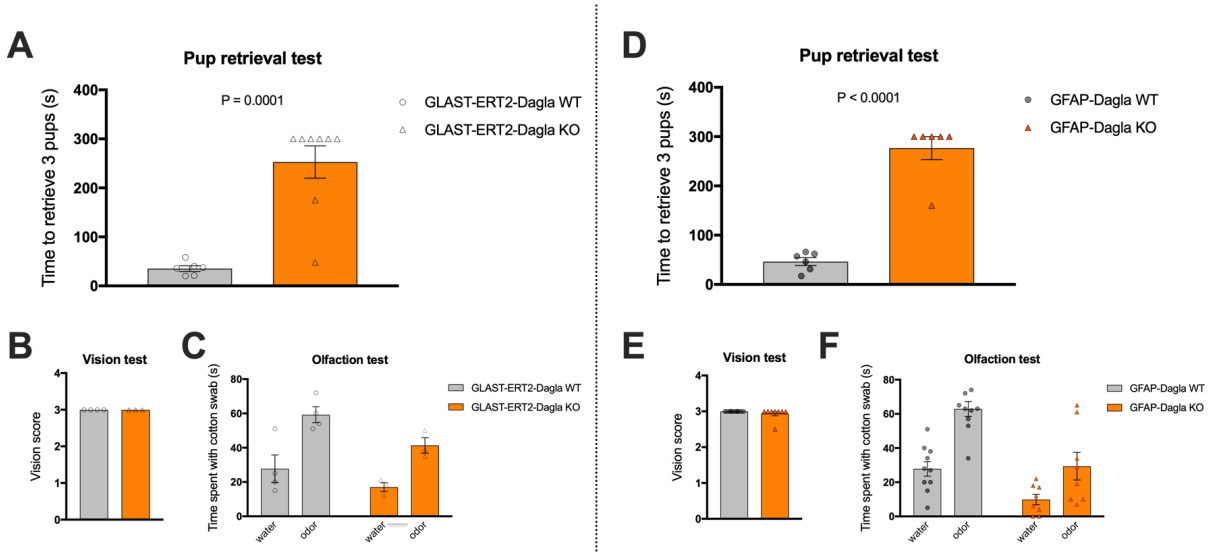


Figure 3.32 GFAP-Dagla and GLAST-ERT2-Dagla KO female mice show impaired maternal care despite functional vision or olfaction.

(A) *GLAST-CreERT2-Dagla KO dams showed impaired maternal care behavior in the pup retrieval test; the time to retrieve three pups was significantly increased. Two of three GLAST-CreERT2-Dagla KO mothers did not retrieve the pups during the five-minute testing period, whereas tamoxifen-injected GLAST-CreERT-Dagla WT dams retrieved the pups immediately.* ***(B)*** *Vision was not affected by GLAST-CreERT2-Dagla KO; all the mice were graded with the best score (3).* ***(C)*** *GLAST-CreERT2 KO mice spent significantly more time with the scented cotton swab (discrimination between water/odor: $F_{1,5}$=75.98, p=0.0003; genotype effect: $F_{1,5}$=3.465, p=0.1217), suggesting proper olfaction.* ***(D)*** *GFAP-Dagla KO dams also displayed impaired maternal care in the pup retrieval test. Five of six GFAP-Dagla KO dams did not retrieve the pups in five minutes without showing impairments in* ***(E)*** *vision or* ***(F)*** *olfaction (discrimination between water/odor: $F_{1,16}$=52,7; p<0.0001; genotype effect: $F_{1,16}$=17.11, p=0.0008).* ***(G)*** *Syn-Dagla KO dams (specific deletion of Dagla in neurons) did not show impairments in maternal care. All tested animals retrieved three of their pups in less than two minutes. Values represent mean ± SEM; n = 3-10 animals/group.*

GFAP-Dagla KO dams showed a very similar phenotype to GLAST-CreERT2-Dagla KO dams in the pup retrieval test. They also needed much longer than GFAP-Dagla WT control dams to retrieve the pups, with several not retrieving any of the pups within the five-minute testing period (Figure 3.32D). GFAP-Dagla KO mice showed no difference in the visual performance and they were able to discriminate between the two cotton swabs in the olfaction test (Figures 3.32E and F). However, they spent less time with the cotton swabs compared to GFAP-Dagla WT controls (two-way ANOVA; genotype effect: p = 0.0008; differentiation water/odor: p = 0.0001).

To investigate if hormones involved in maternal care are generally expressed differently in our mouse model, we performed a transcription analysis in hypothalamus of female GFAP-Dagla KO mice using quantitative real-time PCR (Figure 3.33A,B,C). We found that neither mRNA transcripts of oxytocin nor vasopressin were changed in the hypothalamus of GFAP-Dagla KO mice (Figure 3.33A,B). Since maternal care behavior can also be changed due to stress and/or dysregulations of the HPA-axis, we also analyzed the expression of corticosterone-releasing hormone in the hypothalamus of female GFAP-Dagla KO mice that was also not altered. Determination of CORT in

feces during the period of 24 hours of female mice by ELISA revealed a slight but not significant difference in CORT concentrations between KO and control mice (Figure 3.33D).

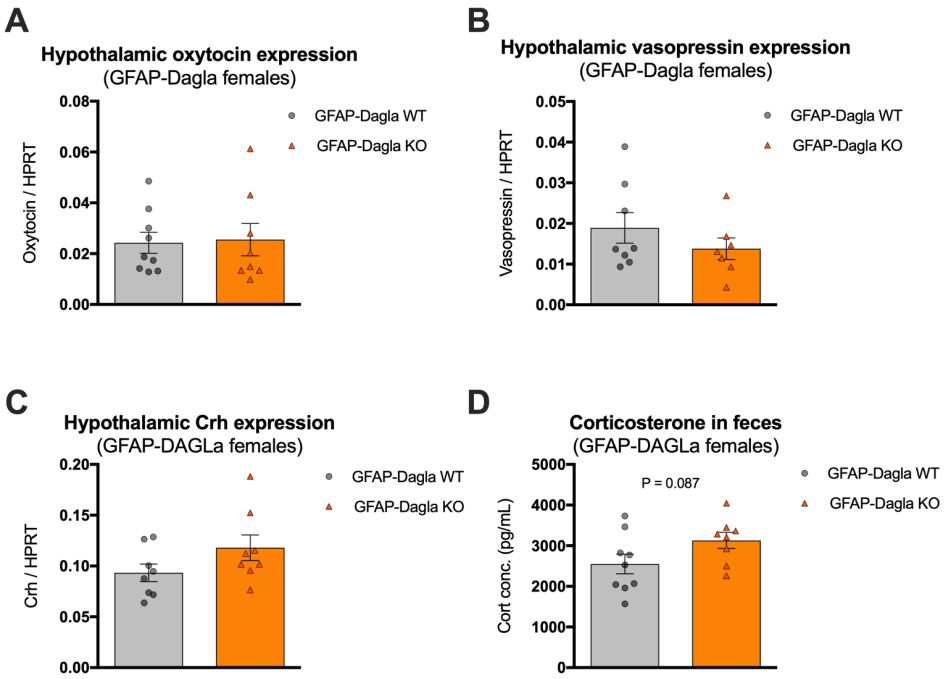


Figure 3.33 Analysis of components connected to maternal care in female GFAP-Dagla mice.

(A) Oxytocin mRNA levels in hypothalamus of female GFAP-Dagla KO mice were not significantly changed. (B) Vasopressin mRNA levels in hypothalamus of female GFAP-Dagla KO mice were also not altered. (C) Corticosterone-releasing hormone (Crh) mRNA levels were slightly elevated but not significantly different in hypothalamus of female GFAP-Dagla KO mice. (D) Analysis of the steroid hormone corticosterone by ELISA showed a tendency for increased stress hormone in the feces of GFAP-Dagla KO mice. Values represent mean ± SEM; n = 8-10 animals/group.

Our findings strongly indicate that astrocytic 2-AG is important for maternal care behavior.

3.2.6 Adult neurogenesis in astrocyte-specific Dagla KO mice

Changes in adult neurogenesis were evaluated by counting BrdU positive cells in the subgranular zone of the dentate gyrus in GFAP-Dagla KO, GLAST-CreERT2-Dagla KO mice, and littermate controls. One day after BrdU injections one cohort of mice was perfused and their brains were stained with an antibody against BrdU. BrdU-positive cells in the dentate gyrus were compared between GFAP-Dagla KO and controls to detect changes in proliferation of progenitor cells. Indeed, GFAP-Dagla KO mice showed a significantly reduced number of BrdU-positive cells in the dentate gyrus, suggesting decreased proliferation (Figure 3.34B). To evaluate changes in survival and differentiation of the newly formed cells, another cohort of mice was perfused 21 days after BrdU injections, brains were stained and BrdU positive cells were counted. Additionally, brains were stained with antibodies against neuronal (NeuN) and astrocytic markers (GFAP & S100β) to

investigate the cell fate of BrdU-positive progenitor cells. 21 days after the last BrdU injection, GFAP-Dagla KO mice showed a lower number of BrdU-positive cells compared to littermate controls in the dentate gyrus (Figure 3.34B). Differentiation of progenitor cells seemed not to be changed since almost the same percentage of BrdU positive cells were positive for neuronal or astrocytic markers (Figure 3.34C).

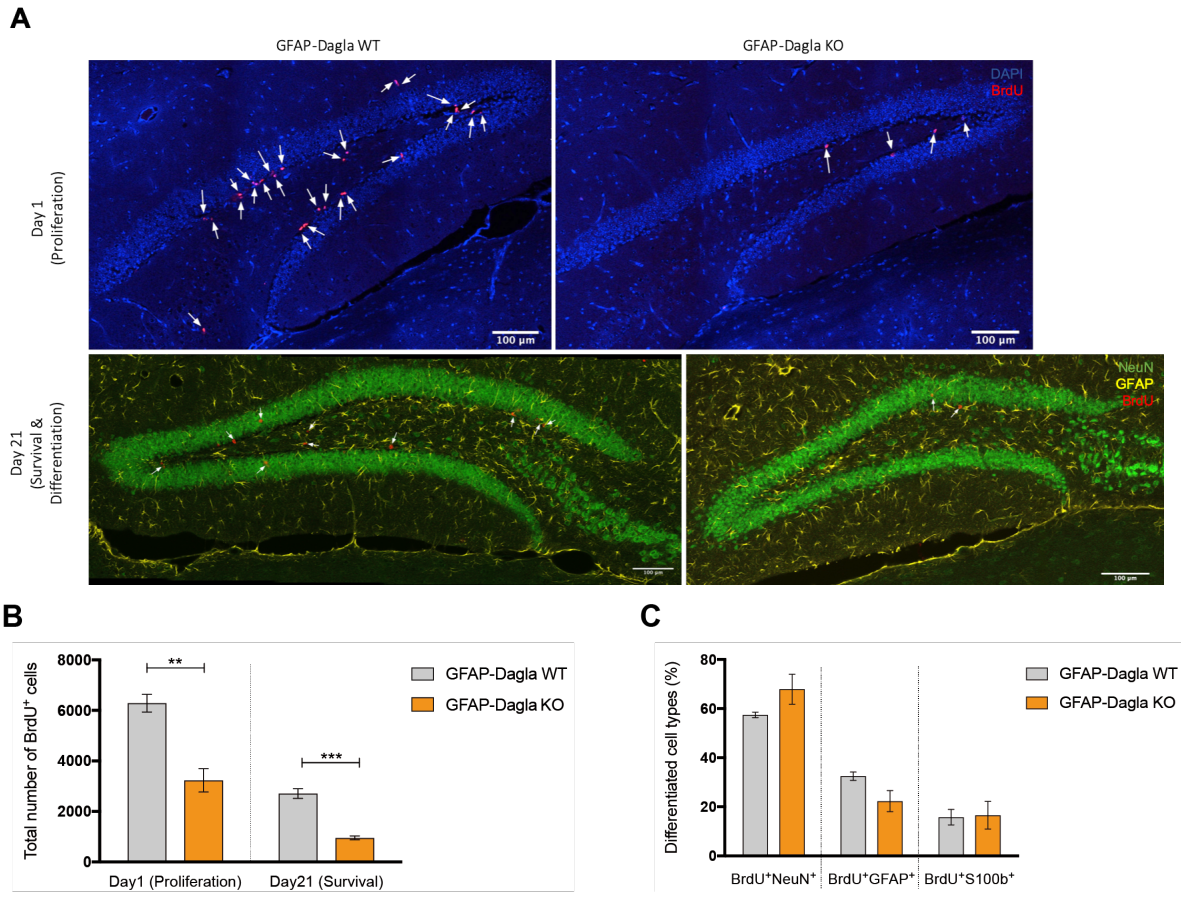


Figure 3.34 Adult hippocampal neurogenesis in GFAP-Dagla mice

GFAP-Dagla KO mice and control mice were treated with intraperitoneal injections of BrdU (50 mg/kg) for 3 consecutive days, mice were perfused 24 h (for proliferation) or 21 days (for survival and differentiation) after the last BrdU injection. ***(A)*** *Representative immunohistochemistry micrographs of GFAP-Dagla KO and control mice. GFAP-Dagla KO mice show less BrdU-positive cells (red) in the dentate gyrus, 1 day and also 21 days after BrdU injections. After 21 days the brains were additionally stained with an astrocytic marker GFAP (yellow) and a neuronal marker NeuN (green) to investigate differentiation. Scale bar: 100 µm.* ***(B)*** *BrdU-positive cells in dentate gyrus of GFAP-Dagla KO mice were significantly reduced 1 day ($p=0.002$) and 21 days ($p=0.0001$) after BrdU injections.* ***(C)*** *Analysis of differentiation of progenitor cells in dentate gyrus of GFAP-Dagla KO mice did not show any differences. BrdU-positive cells were analyzed for co-expression of neuronal marker NeuN and astrocytic markers GFAP and S100β. Values represent mean ± SEM; n = 4 animals/group, 6 analyzed pictures/animal.*

Similarly, GLAST-CreERT2-Dagla KO mice also showed decreased proliferation of progenitors, measured by comparing the number of BrdU-positive cells at day one. 21 days after BrdU injections, the number of BrdU-positive cells was still decreased in KO mice compared to their littermate controls (Figure 3.35B). The differentiation of progenitor cells was unaltered in GLAST-CreERT2-

Dagla KO mice; the same percentage of BrdU cells differentiated into neurons and astrocytes as in the WT controls (Figure 3.35C).

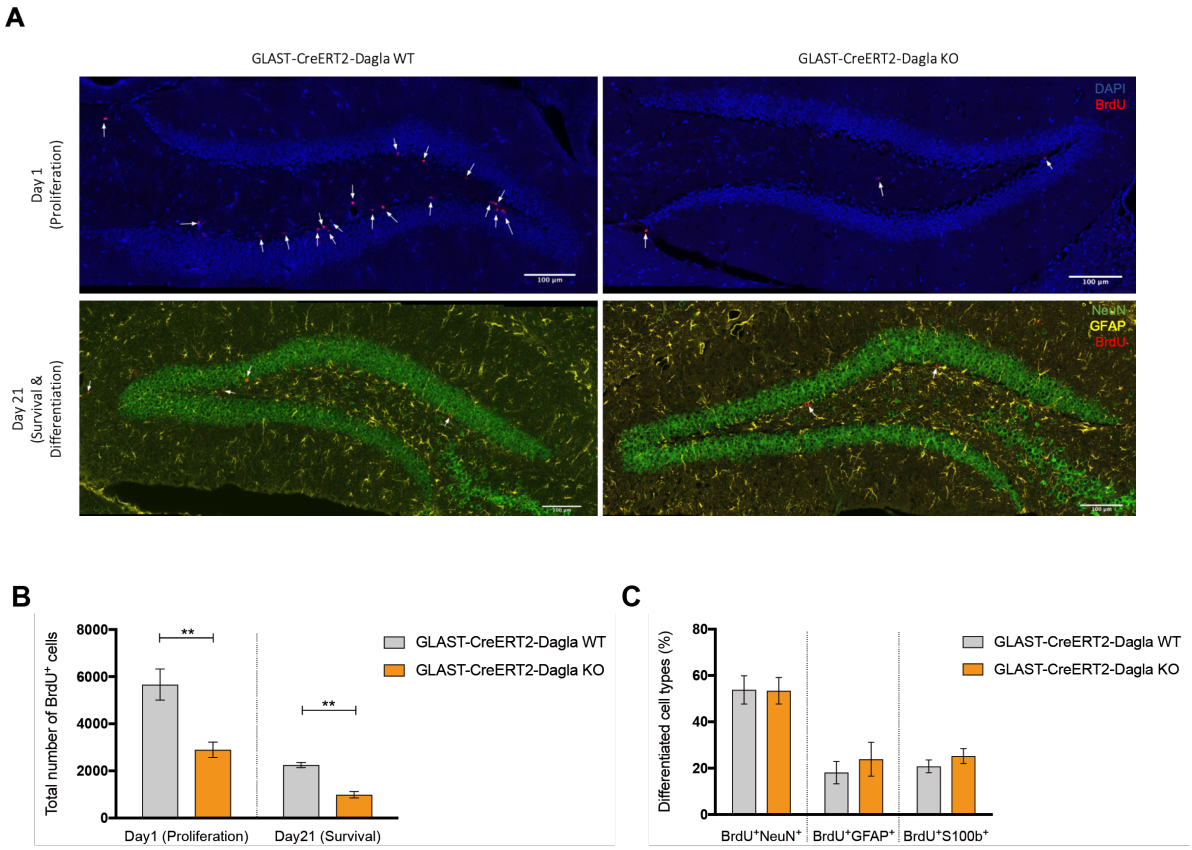


Figure 3.35 Adult hippocampal neurogenesis in GLAST-CreERT2-Dagla mice.

(A) *Representative immunohistochemistry micrographs of GLAST-CreERT2-Dagla WT and KO mice one or 21 days after BrdU injections in dentate gyrus to study proliferation and survival/differentiation, respectively (blue: DAPI; red: BrdU; green: NeuN; yellow: GFAP; scale bar: 100 µm).* ***(B)*** *The number of BrdU-positive cells in dentate gyrus of GLAST-CreERT2-Dagla KO mice was significantly lower compared to WT controls one day (p=0.0095) as well as 21 days (p=0.0003) after BrdU injections.* ***(C)*** *To analyze differentiation of progenitor cells, co-expression of BrdU-positive cells with neuronal marker (NeuN) or astrocytic marker (GFAP and S100) on day 21 were quantified. There were no changes in differentiation between GLAST-CreERT2-Dagla KO and WT control mice. Values represent mean ± SEM; n = 4 animals/group; 6 analyzed pictures per animal.*

It is noteworthy that both, GLAST- and GFAP-promoters, are active in astrocytes, as well as in neural progenitor cells of adult mice (J. Zhang and Jiao 2015). Thus, it might be possible that the DAGLa KO in the progenitor cells themselves might influence neurogenesis in our mouse lines. Using our GLAST-CreERT2-tdTomato reporter line, we wanted to clarify, if and how efficiently Cre recombinase was expressed in neural progenitor cells or in different populations of GLAST-positive cells in the dentate gyrus (Figure 3.36). Therefore, immunohistochemical staining against the progenitor marker GFAP and the proliferation marker Ki67 were performed (Figure 3.36). Analysis showed that Cre-positive cells were mostly expressing GFAP, are located in the subgranular zone, and show the typical shape of a NSC. Furthermore, around 17% of all Cre-expressing (tdTomato positive) cells

in the hilus of dentate gyrus were Ki67 positive, while approximately 76% of all proliferating cells (Ki67 positive) showed Cre expression (data not shown). These data suggest that other cells than neural progenitors also express Cre in the hilus region, however, almost all progenitor cells express Cre recombinase.

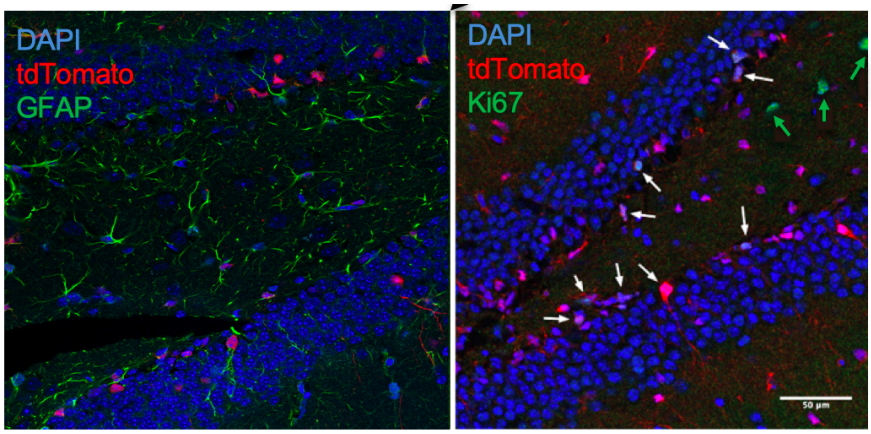


Figure 3.36 Progenitor cells in dentate gyrus of GLAST-CreERT2-Dagla KO mice are expressing Cre recombinase.

Representative immunohistochemistry stainings of dentate gyrus region of GLAST-CreERT2-RosaTomato mouse. Left: Most of the Cre expressing cells (red) are located in the subgranular zone of dentate gyrus, they co-express the progenitor marker GFAP (green), and exhibit the typical shape of a neural stem cell (NSC). Right: Mitotic marker Ki67(green) represents dividing cells (progenitor cells) in dentate gyrus, while tdTomato staining (red) represents Cre recombinase expressing cells. DAPI is shown in blue and scale bar is 50 µm. 17% of all Cre expressing cells in dentate gyrus are progenitor cells, whereas 76% of all progenitor cells in dentate gyrus express Cre recombinase. Values represent mean ± SEM; n = 3.

Next, the expression of different neurogenic factors was investigated in the hippocampus of astrocyte-specific Dagla KO mice using RT-PCR (Figure 3.37). *Dagla* mRNA was significantly downregulated in the hippocampus of GFAP-Dagla KO mice compared to controls, whereas expression of *Dagla* in the hippocampus of GLAST-CreERT2-Dagla mice was not significantly lower. *Daglb* and *CB1* expression were unchanged in both mouse lines. Brain-derived neurotrophic factor (*BDNF*) mRNA levels were not altered in neither GLAST-CreERT2- nor GFAP-Dagla KO mice in comparison to Dagla fl/fl control mice. Hippocampal mRNA levels of the vascular and endothelial growth factor (*VEGF*) were not different in GFAP- and GLAST-CreERT2-Dagla KO mice compared to controls. The expression levels of the cytokine interleukin 1-beta (*IL-1b*), upregulation of which is known to decrease adult neurogenesis (Kaneko et al. 2006), were also not changed in either of the astrocytic lines compared to control Dagla fl/fl mice. Fibroblast growth factor 2 (*FGF2*) is known to promote proliferation of neural progenitor cells in the adult brain (Mudò et al. 2009). The expression of this factor was significantly reduced in the hippocampus of GFAP-Dagla KO mice as well as in GLAST-CreERT2-Dagla KO mice in comparison to control mice. The expression level of insulin-like growth factor (*IGF2*), that controls proliferation of dentate gyrus neural stem cells (NSCs) through AKT-dependent signaling (Bracko et al. 2012), was unchanged in GFAP-Dagla KO mice but tendentially decreased in astrocyte-specific GLAST-CreERT2-Dagla KO mice. Glucocorticoid

receptor (*GR*) mRNA expression levels in the hippocampus were similar in GFAP-Dagla KO mice, GLAST-CreERT2-Dagla KO and control Dagla fl/fl mice.

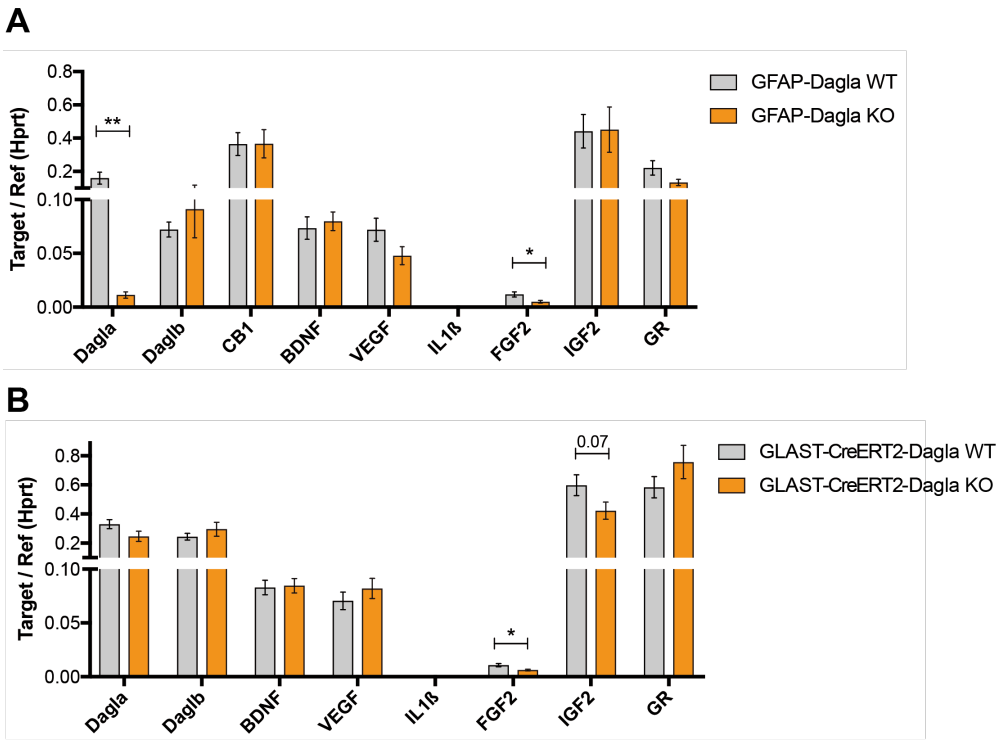


Figure 3.37: Transcriptional analysis of genes involved in adult neurogenesis or the endocannabinoid system in the hippocampus of astrocytic Dagla KO mice.

(A) *Dagla transcription was reduced in hippocampus of GFAP-Dagla KO mice compared to controls (p=0.005). Expression of Daglb and CB1, brain derived neurotropic facto (BDNF), vascular and endothelial growth factor (VEGF), interleukin 1-beta (IL1β), insulin-like growth factor 2 (IGF2) and glucocorticoid receptor (GR) was not changed. Fibroblast growth factor 2 (FGF2) mRNA transcripts were significantly lower compared to control littermates (p=0.04). Values represent mean ± SEM; n=7-9 animals/group.* ***(B)*** *Expression of Dagla in the hippocampus of GLAST-CreERT2-Dagla KO mice was not significantly lower compared to littermate controls (p=0.09). Transcripts of Daglb, BDNF, VEGF, IL1ß, IGF2 and GR were not altered in hippocampus of GLAST-CreERT2-Dagla KO mice. The expression of FGF2 was significantly lower in GLAST-CreERT2-Dagla KO mice compared to control littermates (p=0.015). Values represent mean ± SEM; n=10 animals/group*

3.3 Microglia-specific Cx3Cr1-CreERT2-Dagla and LyzM-Dagla KO mice

To investigate the role of DAGLa in microglia cells, conditional LyzM-Dagla KO and inducible Cx3Cr1-CreERT2-Dagla KO mice together with their littermates were used.

Male Cx3Cr1-CreERT2-Dagla KO and control littermates developed a strong abdominal swelling upon tamoxifen injections (Figure 3.38). This possible hernia was so severe in this line that the

experiment was discontinued in males to avoid any possible suffering. Male mice were not tested in behavioral tests but the brains were prepared for endocannabinoid measurements.

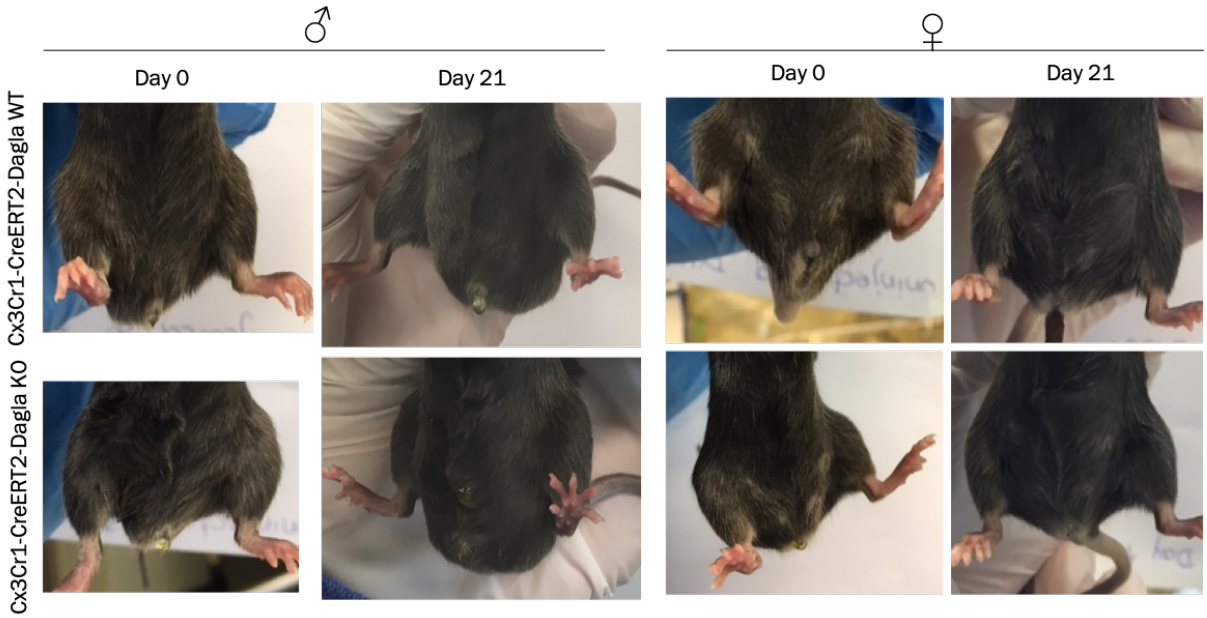


Figure 3.38 Development of tamoxifen-induced abdominal swelling/hernia in male Cx3Cr1-CreERT2-Dagla KO and WT control mice.

21 days after tamoxifen-injections, male but not female mice developed a putative hernia independent of their genotype. Male Cx3Cr1-CreERT2-Dagla KO and WT mice were not used for behavioral testing.

Body weight and the food intake of female Cx3Cr1-CreERT2-Dagla KO mice was unchanged in comparison to their littermate controls (Figure 3.39A). Only one day after the sucrose preference test, female Cx3Cr1-CreERT2-Dagla KO mice ate significantly more food compared to their controls (Figure 3.39B). Similarly, neither male nor female LyzM-Dagla KO mice showed any differences in body weight or food intake compared to their littermate controls (Figure 3.39C-E).

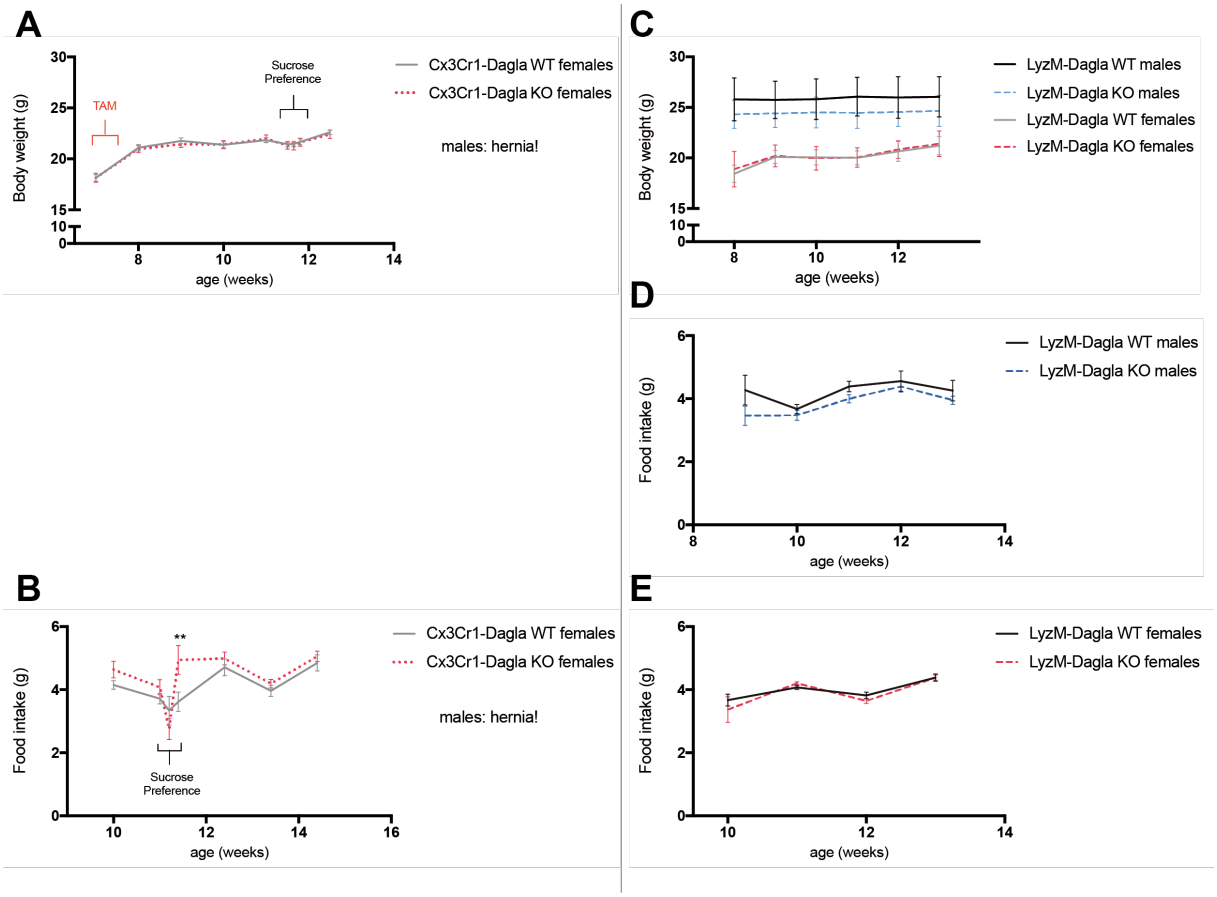


Figure 3.39 Body weight and food intake of Cx3Cr1-CreERT2- and LyzM-Dagla KO mice.

(A) Body weight of female Cx3Cr1-CreERT2-Dagla KO mice was not changed compared to WT controls (genotype effect: $F_{1,26}$=0.05032, p=0.8243; time effect: $F_{8,208}$=155.6, p<0.0001). (B) Cx3Cr1-CreERT2-Dagla KO mice showed no changes in food intake compared to WT controls (genotype effect: $F_{1,26}$=2.119, p=0.1575; time effect: $F_{6,156}$=14.36, p<0.0001). Post-hoc analysis showed that food intake was increased in Cx3Cr1-CreERT2-Dagla KO mice one day after sucrose preference test in comparison to WT controls. (C) Body weight of male and female LysM-Dagla KO mice were unchanged compared to their WT controls. Male (D) and female (E) LyzM-Dagla KO mice did not show significant differences in food intake compared to WT controls (males: genotype effect: $F_{1,13}$=2.2, p=0.1619; time effect: $F_{4,52}$=5.895; p=0.0005).

Additionally, both microglia-specific Dagla KO mouse lines showed normal survival (data not shown).

3.3.1 Dagla expression and endocannabinoid production by microglia

For validation of our microglia-specific KO lines, a DAGLa immunostaining was performed (Figure 3.40A). DAGLa antibody did bind specifically, validated by the absent signal in constitutive Dagla KO brain. In LyzM-Dagla KO mouse brain, DAGLa signal intensity was reduced in the thalamus, CA3 region of hippocampus and partially in the cortex. DAGLa staining in inducible Cx3Cr1-CreERT2-Dagla KO brain was similar to control Dagla fl/fl mice but slightly reduced.

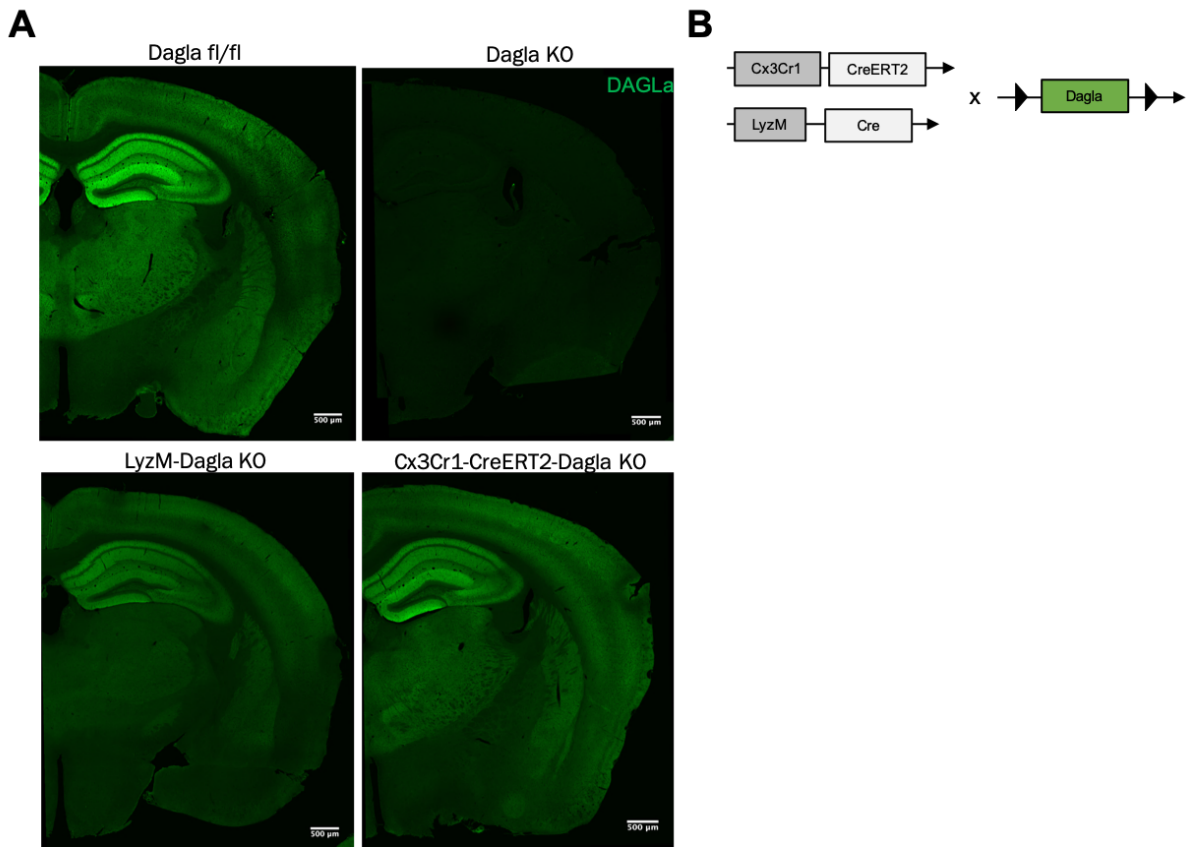


Figure 3.40: Validation of LyzM-Dagla and Cx3Cr1-CreERT2-Dagla KO efficacy by immunohistochemistry

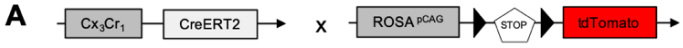
***(A)** Representative images of DAGLa immunostainings from brains of Dagla fl/fl control mice, tamoxifen-induced Cx3Cr1-CreERT2-Dagla KO, LyzM-Dagla KO and constitutive Dagla KO control mice. DAGLa staining is completely absent in constitutive Dagla KO mice. In LyzM-Dagla KO mice Dagla signal intensity seems to be reduced in the thalamus and CA3 region of hippocampus. Dagla staining in Cx3Cr1CreERT2-Dagla KO brain looks very similar to the staining in Dagla fl/fl control mice. Scale bar: 500 µm; green: DAGLa (n=3). **(B)** Genetic description of Cx3Cr1- and LyzM-Dagla KO mice.*

For further validation of the promoter efficacy and specificity in our mouse lines, we crossed Cx3Cr1-CreERT2 mice into RosaTomato reporter line (Figure 3.41A). Offspring from this breeding does express the red fluorescing protein tdTomato in all Cre recombinase expressing cells. An immunohistochemical staining for microglia marker Iba1 was performed to identify the co-expression of Iba1 with tdTomato (Figure 3.41C). The analysis was performed by PD Dr. Andras Bilkei-Gorzo.

Cx3Cr1 promoter was very specific for microglia cells (% of Cre positive cells that were also Iba1 positive) in the cortex (92%), hippocampus (98%) and hypothalamus (94%) (Figure 3.41B). 96% of all Iba1 positive cells in the cortex, 94% in the hippocampus and 88% in the hypothalamus expressed tdTomato, suggesting a very high promoter efficacy (Figure 3.41B).

These results suggest a very specific and efficient Dagla KO in microglial cells in our Cx3Cr1-CreERT2-Dagla KO mice. Unfortunately, tdTomato expression was also found in microglia of Cx3Cr1-CreERT2-Dagla KO mice without tamoxifen injections, which indicates that the KO was actually not inducible.

A



B

Cx3Cr1-CreERT2-Dagla

Area	Promoter specificity (%)	Promoter efficacy (%)
Cortex	92.28 ± 9.45	96.47 ± 6.18
Hippocampus	98.39 ± 1.64	94.16 ± 6.39
Hypothalamus	93.78 ± 5.62	88.24 ± 10.87

C

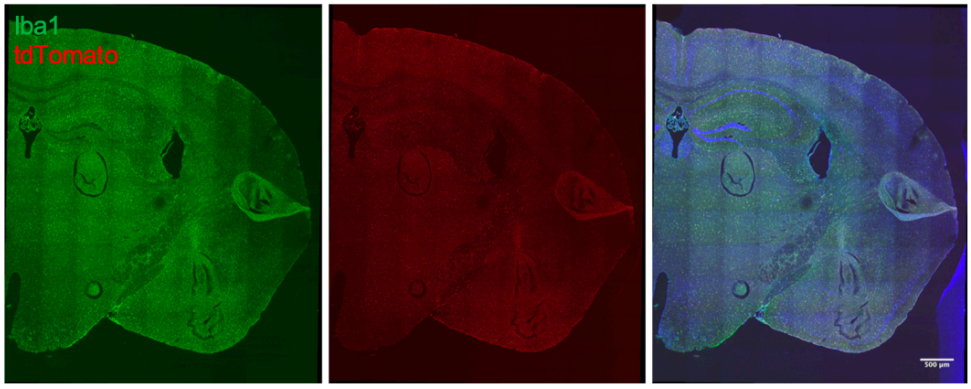


Figure 3.41 Validation of Cre expression in Cx3Cr1-CreERT2-Dagla KO mice by Rosa-Tomato reporter line.

(A) *Genetic description of mice used for validation of Cre expression.* ***(B)*** *Quantification of Cre expression efficacy in microglia (% of tdTomato cells co-expressing Iba1 from all Iba1-positive cells) and cell-type specificity (% of cells co-expressing tdTomato and microglial marker Iba1 from all Cre-expressing cells) in different brain regions of Cx3Cr1-CreERT2-RosaTomato mice* ***(C)*** *Representative immunohistochemistry picture of a Cx3Cr1-CreERT-RosaTomato mouse with microglia-marker Iba1 (green) and tdTomato (red) and a merged image (scale bar: 500 µm; n=3 per group, 4 of sections per animal for quantification). Rosa-Tomato- quantification was performed by PD Dr. Andras Bilkei-Gorzo, University of Bonn.*

The same procedure was applied for LyzM-Dagla KO mice. Upon crossing LyzM-Cre mice into RosaTomato reporter mouse line, we found that only 2-3% of all Cre expressing cells in the hippocampus and cortex were positive for the microglial marker Iba1 (Figure 3.42B). Furthermore, only 0-11% of all microglia cells did express Cre in these regions, suggesting a very high off-target effect of the LyzM-promoter. To identify which population expressed the off-target Cre, LyzM-RosaTomato mouse brains were immunohistochemically stained with an antibody against the neuronal marker NeuN (Figure 3.42E). In the cortex and hippocampus 91% of all Cre expressing cells were positive for the NeuN. However, only 15-17% of all NeuN-positive neurons in those regions did express tdTomato. Altogether, these data suggest that LyzM-promoter is almost not active in microglia cells. Instead, LyzM-promoter seems to be very specific for neurons, however, it affects only a subpopulation of neurons.

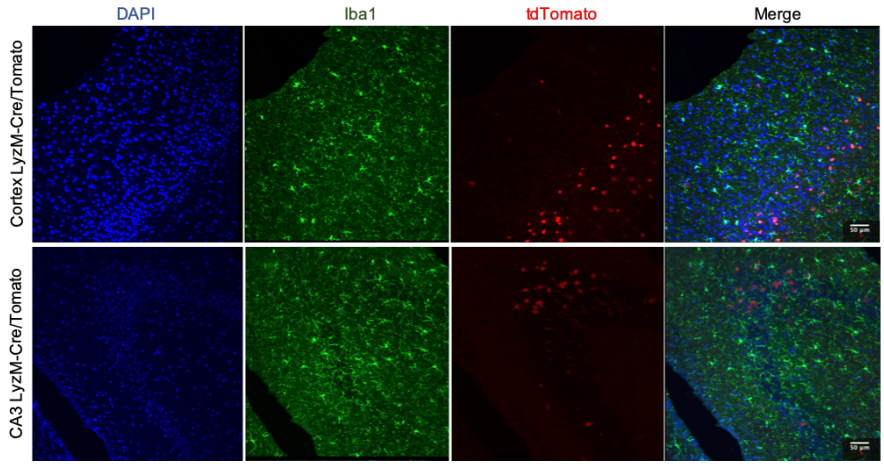
A

B

LyzM-Dagla

Area	Approx. promoter specificity (%) in MG	Approx. promoter efficacy (%) in MG
Cortex	3.95 ± 6.53	11.15 ± 11.13
Hippocampus	2.02 ± 4.78	0.33 ± 0.98

C



D

LyzM-Dagla

Area	Approx. promoter specificity (%) in Neurons	Approx. promoter efficacy (%) in Neurons
Cortex	91.06 ± 3.95	16.67 ± 5.81
Hippocampus	91.43 ± 6.89	15.46 ± 11.28

E

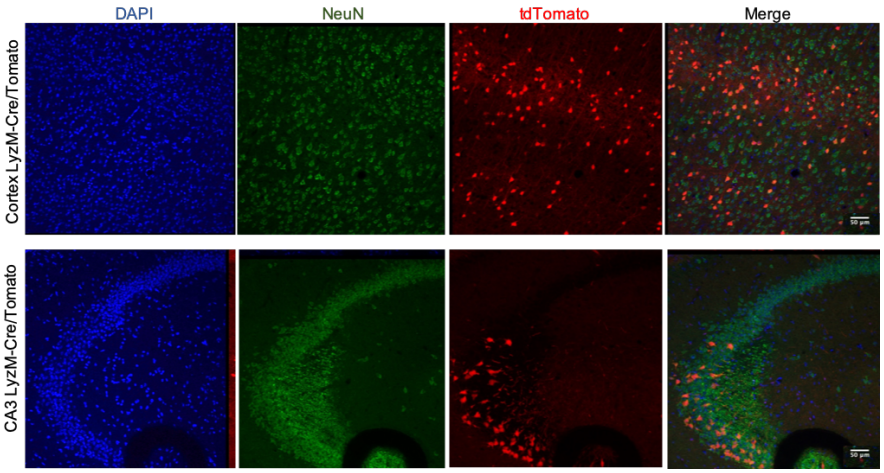


Figure 3.42 Validation of Cre expression in LyzM-Dagla KO mice by Rosa-Tomato reporter line.

(A) *Genetic description of mice used for validation of Cre expression.* ***(B)*** *Quantification of Cre expression efficacy in microglia (MG) (% of tdTomato cells co-expressing Iba1 from all Iba1-positive cells) and cell-type specificity (% of cells co-expressing tdTomato and microglial marker Iba1 from all Cre-expressing cells) in different brain regions of LyzM-RosaTomato mice* ***(C)*** *Representative immunohistochemistry picture of a LyzM-RosaTomato mouse cortex (top) and hippocampal CA3 region (bottom) with microglia-marker Iba1 (green) and tdTomato (red). No co-localization of tdTomato and microglial marker Iba1 was found in the cortex and hippocampus of LyzM-Cre mice.* ***(D)*** *Quantification of Cre expression efficacy in neurons (% of tdTomato cells co-expressing NeuN from all NeuN-positive cells) and cell-type specificity (% of cells co-expressing tdTomato and neuronal marker NeuN from all Cre-expressing cells) in different brain regions of LyzM-RosaTomato mice.* ***(E)*** *Representative immunohistochemistry picture of a LyzM-RosaTomato mouse cortex (top) and hippocampal CA3 region (bottom) with neuronal-marker NeuN (green) and representing tdTomato (red). tdTomato signal is overlapping with neuronal marker NeuN in*

cortex and of LyzM-Cre mice. (Scale bar: 500 um; n=3 per group, 4 of sections per animal for quantification). Rosa-Tomato- analysis (Table) was performed by PD Dr. Andras Bilkei-Gorzo, University of Bonn.

In order to investigate the contribution of microglial cells to 2-AG production, different brain regions were isolated for targeted lipidomics performed by our collaboration partners in Bern. Neither male nor female microglia-specific Cx3Cr1-CreERT2-Dagla KO mice showed any changes in 2-AG levels in the tested brain regions. The levels of 2-AG precursor SAG were also not altered in male and female Cx3Cr1-CreERT2-Dagla KO mice compared to control littermates. The levels of AA, which is the metabolite of 2-AG, were unchanged as well.

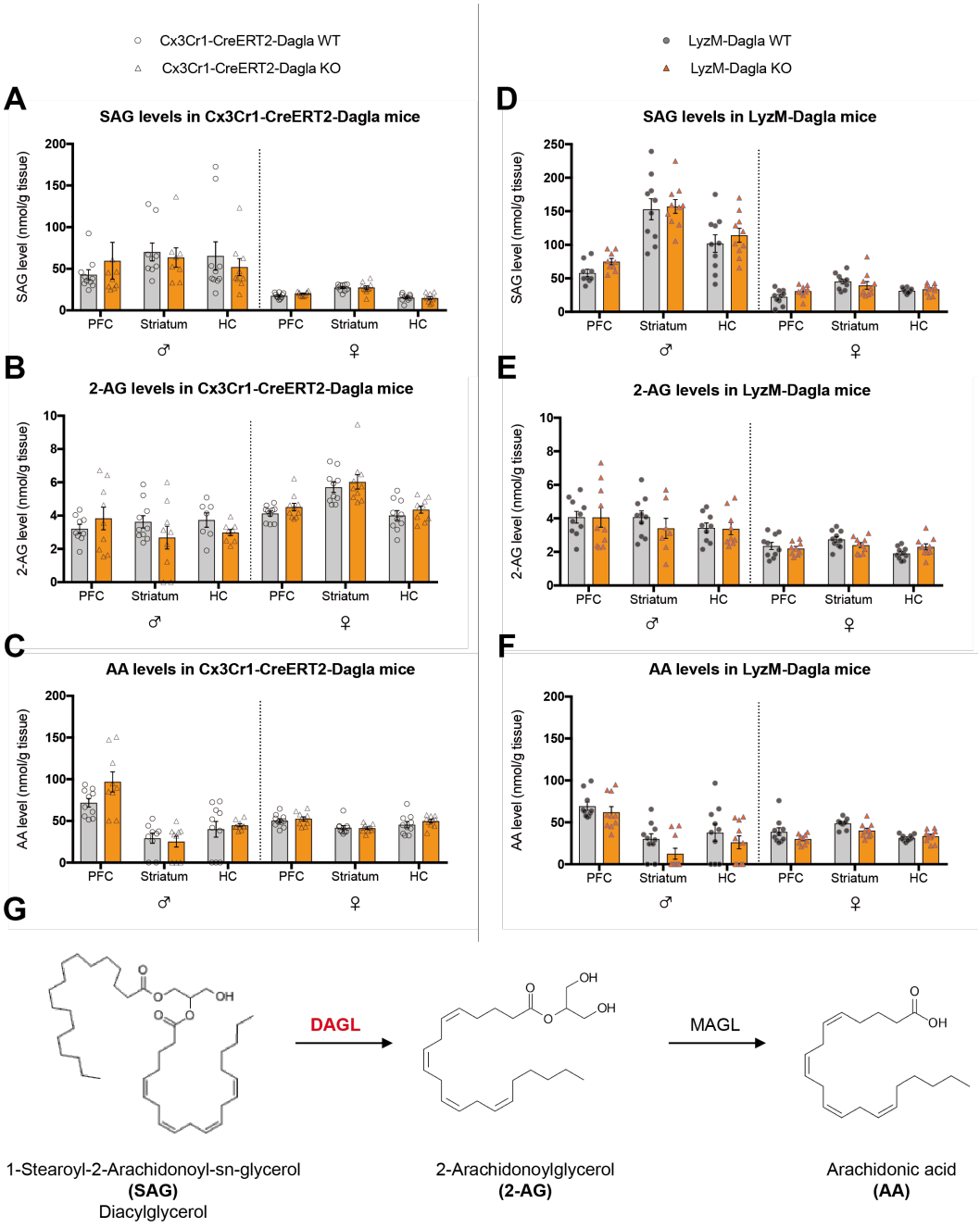


Figure 3.43: Measurements of components of the endocannabinoid system by LC-MS/MS in microglia-specific Dagla KO mice.

(A) *The substrate of DAGLa, 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG), was not significantly changed in any of the tested brain regions (prefrontal cortex (PFC), striatum, hippocampus (HC) and hypothalamus (HY) in male or female Cx3Cr1-CreERT2-Dagla mice.* ***(B)*** *The levels of the endocannabinoid 2-arachidonoylglycerol (2-AG) were unchanged in all tested brain regions in Cx3Cr1-CreERT2-Dagla KO mice compared to littermate controls, as well as the metabolite of 2-AG, arachidonic acid (AA)* ***(C). (D, E, F)*** *LyzM-Dagla KO mice did not show any changes in SAG, 2-AG or AA in any of the tested brain regions compared to control mice.* ***(G)*** *Schematic representation of the synthesis and metabolism of 2-AG by the enzymes DAGL and monoacylglycerol lipase (MAGL). Values represent mean ± SEM; n (Cx3Cr1) = 10 animals/group; n (LyzM) = 8-10 animals/group. Measurements were performed by Sandra Glasmacher, University of Bern.*

LyzM-Dagla KO mice of both sexes neither showed changes in SAG, 2-AG nor in AA levels compared to controls.

3.3.2 Exploratory and anxiety-related behavior of Cx3Cr1-CreERT2-Dagla and LyzM-Dagla mice

Exploratory and anxiety-related behaviors of Cx3Cr1-CreERT2- and LyzM-Dagla KO mice were assessed in the open-field and the zero-maze tests. Male Cx3Cr1-CreERT2-Dagla KO mice were not tested in behavioral tests as mentioned above because they developed a severe tamoxifen-induced abdominal swelling/hernia. Female Cx3Cr1-CreERT2-Dagla KO mice did not show an anxiety-related phenotype in the open-field test, measured by the time spent in the center of the box. They also traveled a similar distance in the open-field box compared to littermate controls (Figure 3.44A). In the zero-maze test, female Cx3Cr1-CreERT2-Dagla KO mice spent slightly less time (p=0.066) in the open compartment of the maze and traveled a smaller distance in the maze in comparison to controls. Female Cx3Cr1-CreERT2-Dagla KO mice tended to look down from the maze less often (p=0.060) and showed a stretched posture when leaving the closed compartment significantly more often than littermate controls (Fig 3.44C). All behavioral changes in the zero-maze suggest increased anxiety of this mouse line (Shepherd et al. 1994).

LyzM-Dagla KO mice showed normal behavior in the open-field test (Figure 3.44B). In the zero-maze test, time spent in the open-compartment and distance moved were similar in both male and female LyzM-Dagla KO mice to their littermate controls. Female, but not male LyzM-Dagla KO mice were looking down from the maze significantly less often than controls and they stretched their body when leaving the closed compartment more often than control mice (Figure 3.44D). Looking down less often and stretching posture more are considered characteristic of increased anxiety (Shepherd et al. 1994).

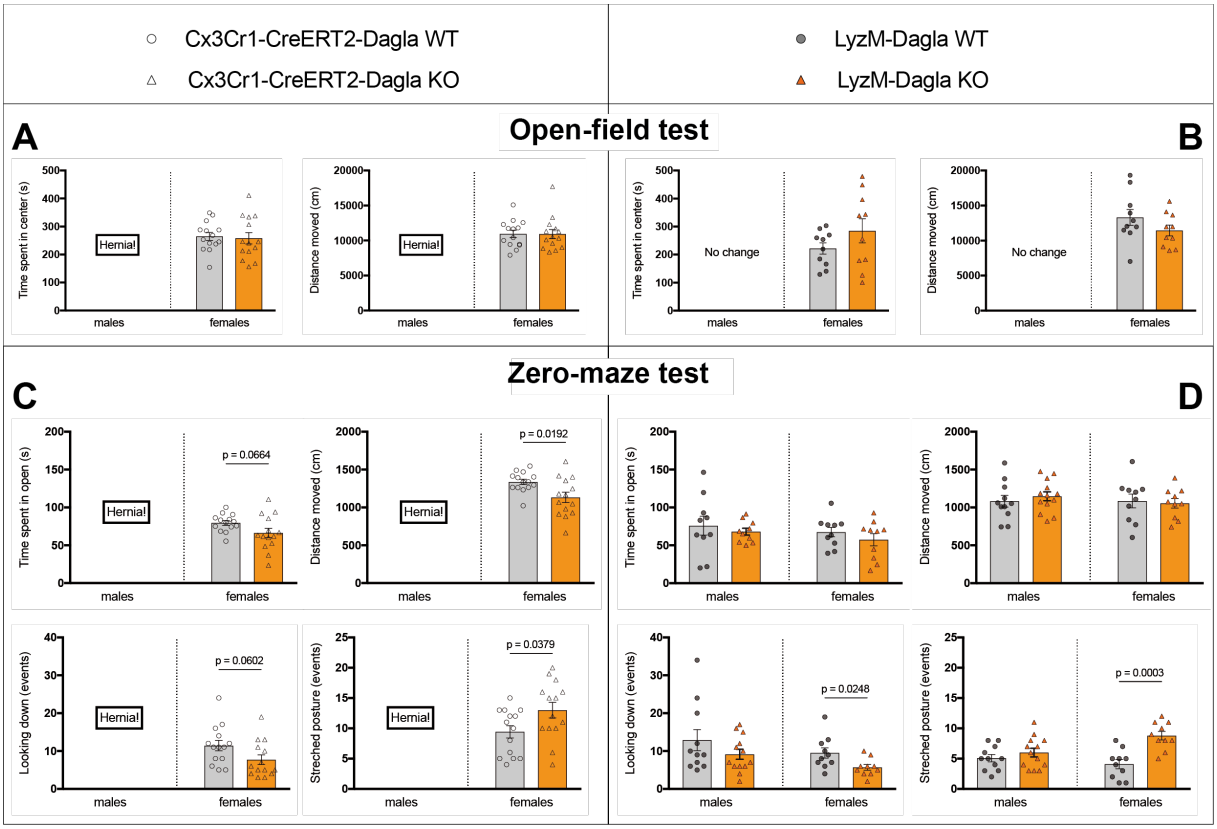


Figure 3.44 Exploratory- and anxiety-related behaviors of microglia-specific Cx3Cr1-CreERT2-Dagla KO and LyzM-Dagla KO mice.

(A) *In the open-field test, female Cx3Cr1-CreERT2-Dagla KO mice did not show any differences in time spent in center or distance traveled compared to littermate controls. Male mice were not tested because of a severe tamoxifen-induced hernia.* ***(B)*** *Female LyzM-Dagla KO showed no changes in behavior in the open-field test compared to control mice. They spent similar time in the center of the box and traveled a similar distance. Male mice were tested by a former Ph.D. student Imke Jenniches, who also did not find any changes in this test.* ***(C)*** *In the Zero-maze test, female Cx3Cr1-CreERT2-Dagla KO mice showed slightly decreased time in the open compartment of the maze (p=0.066). Distance moved was significantly lower in female Cx3Cr1-CreERT2-Dagla KO mice compared to their control mice. Female Cx3Cr1-CreERT2-Dagla KO mice were looking down from the maze less often and exhibited a stretched posture when leaving the closed compartment more often than the controls.* ***(D)*** *Male and female LyzM-Dagla KO mice spend similar time in the open compartments of the maze as control mice and traveled a similar distance. Female, but not male LyzM-Dagla KO mice were looking down from the maze less often and did a stretched posture more often compared to control mice. Mean ± SEM; n (Cx3Cr1-CreERT2) = 14 animals/group; n (LyzM) = 10 animals/group. Students t-test.*

Taken together, female Cx3Cr1-CreERT2- and LyzM-Dagla KO mice displayed a mild anxiety-like phenotype in the zero-maze test, which was more pronounced in Cx3Cr1-CreERT2-Dagla KO line. Additional anxiety tests should be added to confirm this finding.

3.3.3 Depression-like behavior in Cx3Cr1-CreERT2-Dagla and LyzM-Dagla mice

Female Cx3Cr1-CreERT2-Dagla KO mice did not display a depression-like phenotype in the nestlet, sucrose preference or forced swim tests (Figure 3.45A,B,C). They build nests of similar quality and

showed a similar immobility time in the forced swim test compared to their littermate controls. In the sucrose preference test, female Cx3Cr1-CreERT2-Dagla KO mice almost exclusively drank sucrose and showed even higher preference for sucrose than their littermate controls. Neither male nor female LyzM-Dagla KO mice showed changes in any of the depression-related paradigms (Figure 3.45D-F). They build nests of similar quality, preferred the sucrose solution as much as the controls and showed a similar immobility time in the forced swim test. In the forced swim test, male LyzM-Dagla KO mice also did not show a phenotype (tested by Imke Jenniches, Ph.D. Thesis 2016).

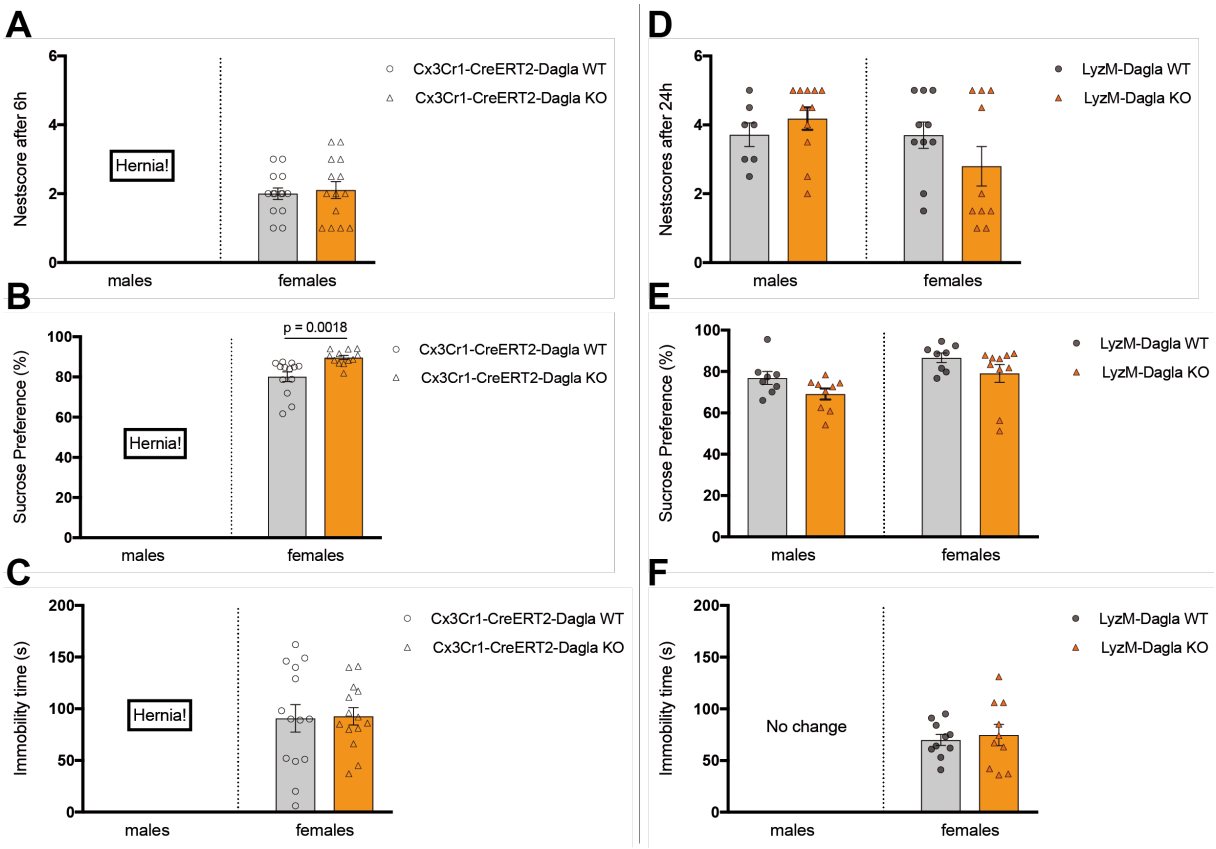


Figure 3.45 Depression-like behavior in Cx3Cr1-CreERT2-Dagla KO and LzyM-Dagla KO mice

*(**A**) Nest-building behavior was unchanged in female Cx3Cr1-CreERT2-Dagla KO mice compared to littermate controls. (**B**) Anhedonia assessed by the sucrose preference test was not changed in female Cx3Cr1-CreERT2-Dagla KO mice. (**C**) Female Cx3Cr1-CreERT2-Dagla KO mice displayed similar immobility in the forced swim test compared to controls. (**D**) Male and female LyzM-Dagla KO mice showed no changes in nest building compared to their controls. (**E**) Sucrose preference was unchanged in both, male and female LyzM-Dagla KO mice. (**F**) In the forced swim test, LyzM-Dagla KO mice of both genders showed a similar immobility time compared to controls. Males were tested by Imke Jenniches. Mean ± SEM; n (Cx3Cr1) = 14 animals/group; n (LyzM) = 10 animals/group. Students t-test.*

In summary, Cx3Cr1-CreERT2-Dagla KO and LyzM-Dagla KO mice do not display a depression-like phenotype.

3.3.4 Maternal care behavior of Cx3Cr1-CreERT2-Dagla and LyzM-Dagla mice

Maternal care behavior was assessed in the pup retrieval test (Figure 3.46). Cx3Cr1-CreERT2-Dagla KO dams did retrieve their pups normally from the opposite corner of the home cage to the nest. Control (Cx3Cr1-CreERT2-Dagla WT) dams were also injected with tamoxifen to exclude possible effects of the substance on maternal care. LyzM-Dagla KO dams showed normal pup retrieval as well. All tested mothers retrieved three pups in less than 1 minute, similar to Dagla fl/fl control dams.

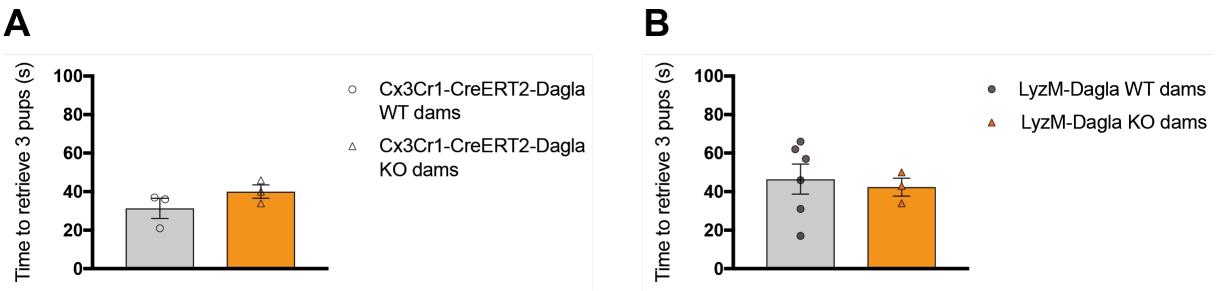


Figure 3.46: Maternal care behavior of Cx3Cr1-CreERT2-Dagla and LyzM-Dagla mice.

***(A)** In the pup retrieval test, Cx3Cr1-CreERT2-Dagla KO dams retrieved the pups in less than one minute to the nest. Cx3Cr1-CreERT2-Dagla WT also retrieved the pups normally. **(B)** LyzM-Dagla KO dams did not show changes in maternal care behavior compared to control dams.*

Taken together, microglial DAGLa KO does not affect maternal care behavior.

3.3.5 Adult neurogenesis in Cx3Cr1-CreERT2-Dagla and LyzM-Dagla mice

Adult hippocampal neurogenesis in Cx3Cr1-CreERT2-Dagla KO and LyzM-Dagla KO mice was investigated by BrdU injections and a subsequent antibody staining against BrdU (Figure 3.47A) as described above. Cx3Cr1-CreERT2-Dagla KO mice showed a similar number of BrdU-positive cells to their controls one day after the last BrdU injection in the dentate gyrus, suggesting no changes in proliferation of neural stem cells in this mouse line (Figure 3.47B). Investigation of survival of progenitor cells 21 days after BrdU injections showed a slightly decreased number of BrdU-positive cells, suggesting reduced survival of progenitor cells in comparison to control mice (Figure 3.47B). Differentiation, however, analyzed by a co-staining with a neuronal (NeuN) and astroglial (GFAP, S100β) markers, was unchanged in Cx3Cr1-CreERT2-Dagla KO mice compared to controls (Figure 3.47C).

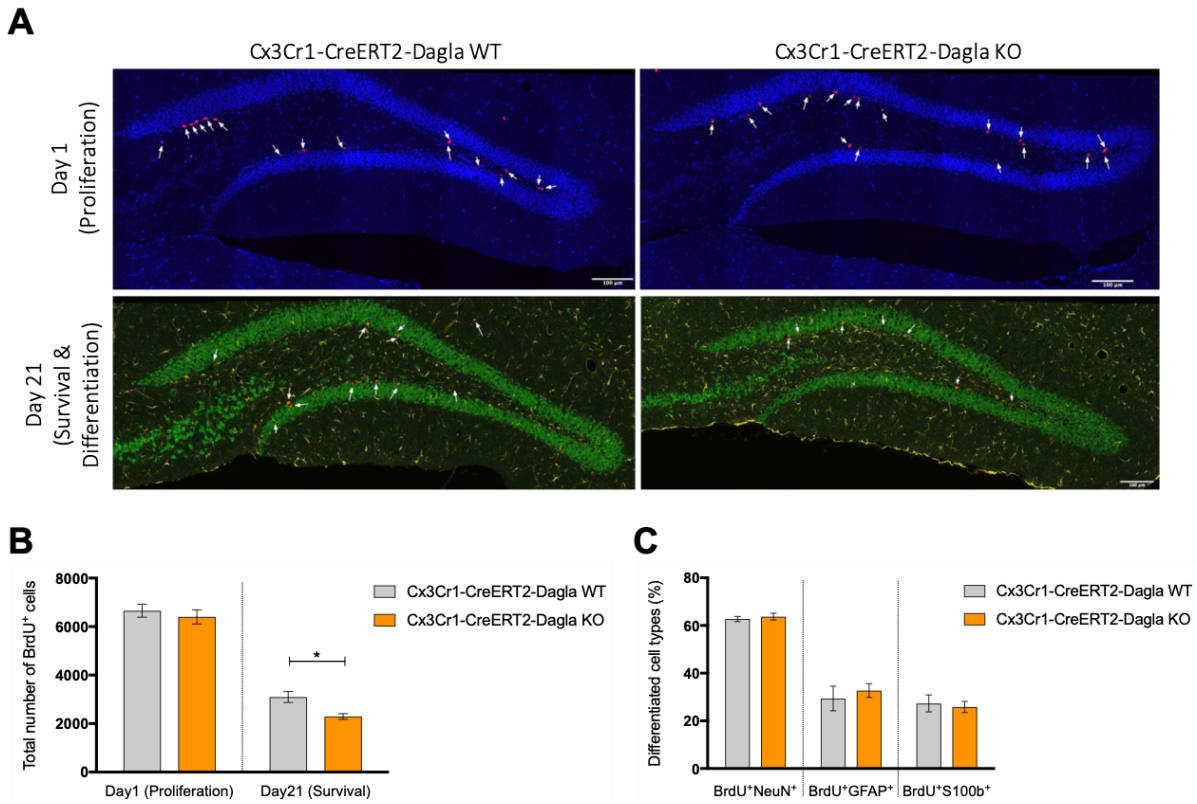


Figure 3.47 Adult hippocampal neurogenesis in Cx3Cr1-CreERT2-Dagla KO mice.

Cx3Cr1-CreERT2-Dagla KO and control mice were treated with intraperitoneal injections of BrdU (50 mg/kg) for 3 consecutive days (one month after tamoxifen treatment). ***(A)*** *Representative immunohistochemistry stainings of Cx3Cr1-CreERT2-Dagla KO and control mice for BrdU-positive cells (red) and Dapi (blue) one day and 21 days after BrdU injections. Additionally, brains were stained with an astrocytic marker GFAP (yellow) and a neuronal marker NeuN (green) on day 21 to investigate differentiation, which was unchanged. Scale bar: 100 µm.* ***(B)*** *BrdU-positive cells in the dentate gyrus of Cx3Cr1-CreERT2-Dagla KO mice are similar to controls one day after BrdU injections. The survival of progenitor cells (day 21) was slightly decreased in Cx3Cr1-CreERT2-Dagla KO mice compared to controls.* ***(C)*** *Analysis of differentiation of progenitor cells in the dentate gyrus of Cx3Cr1-CreERT2-Dagla KO mice. BrdU-positive cells were analyzed for co-expression of neuronal marker NeuN and astrocytic markers GFAP and S100β. Values represent mean ± SEM; n = 4 animals/group, 6 analyzed pictures/animal. Students t-test.*

LyzM-Dagla KO mice showed no differences in the number of BrdU positive cells when checked for proliferation. Survival of progenitor cells was highly reduced in LyzM-Dagla KO mice compared to controls, measured by the number of BrdU positive cells 21 days after the last injection (Figure 3.48B). However, differentiation was unchanged in LyzM-Dagla KO mice. A similar percentage of BrdU-positive cells differentiated into neuronal linage or glial lineage as in the control mice (Figure 3.48C).

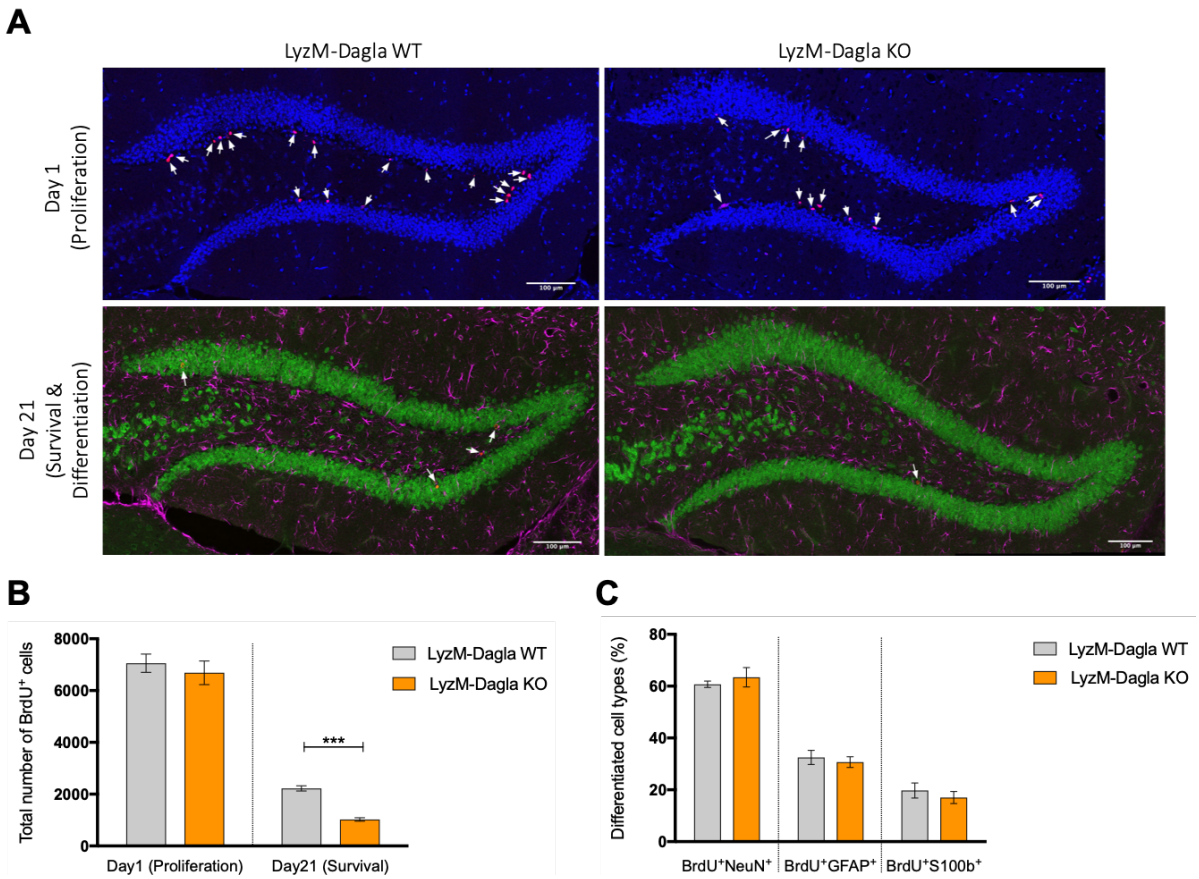


Figure 3.48: Adult hippocampal neurogenesis LyzM-Dagla KO mice.

LyzM-Dagla KO and control mice were treated with BrdU (50 mg/kg) for 3 consecutive days, 24 h and 21 days after the last BrdU injection proliferation and survival/differentiation, respectively, of neural progenitor cells in the dentate gyrus was investigated. ***(A)*** *Representative immunohistochemistry stainings of BrdU-positive cells (red) and Dapi (blue) one day and 21 days after the last injection LyzM-Dagla KO and control mice. Additionally, brains were stained with an astrocytic marker GFAP (magenta) and a neuronal marker NeuN (green) to investigate differentiation, which was unchanged. Scale bar: 100 µm.* ***(B)*** *The number of BrdU-positive cells in the dentate gyrus of LyzM-Dagla KO mice was similar to controls 1 after BrdU injections. Survival of progenitor cells (day 21) was strongly decreased in LyzM-Dagla KO mice compared to controls.* ***(C)*** *Analysis of differentiation of progenitor cells in the dentate gyrus of LyzM-Dagla KO mice. BrdU-positive cells were analyzed for co-expression of neuronal marker NeuN and astrocytic markers GFAP and S100β. Values represent mean ± SEM; n = 4 animals/group, 6 analyzed pictures/animal. Students t-test.*

In conclusion, a deletion of Dagla in microglia/myeloid cells seems to affect survival of progenitor cells in the dentate gyrus of adult mice.

To clarify the mechanism behind decreased survival of progenitor cells in microglia/monocyte-specific Dagla KO mice, transcription analysis of different cytokines was performed. It is known that increased inflammation, for example in obesity, can lead to a reduction of adult neurogenesis (Bracke et al. 2019). Since 2-AG has anti-inflammatory properties (Hillard 2018), we hypothesized that a *Dagla* KO in myeloid cells and a connected decrease in 2-AG levels in the blood could lead to peripheral inflammation. According to literature, LyzM-Dagla KO mice show a high deletion of *Dagla* in myeloid cells and peripheral organs like the liver (Orthgiess et al. 2016). Thus, we used un-perfused liver tissue containing blood for the mRNA transcription analysis. Indeed, *Dagla* mRNA

levels were significantly decreased in liver tissue of LyzM-Dagla KO mice (Figure 3.49A), whereas *Daglb* expression was not different from control mice (Figure 3.49B). Expression of tumor necrosis factor alpha (TNFa), a cytokine involved in inflammation released by macrophages, was not altered in LyzM-Dagla KO mice. mRNA levels of pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1beta (IL-1β) were significantly reduced in LyzM-Dagla KO mice compared to controls, opposing our hypothesis. Transcription of interleukin-10 (IL-10), which has anti-inflammatory properties, was upregulated in liver of LyzM-Dagla KO mice.

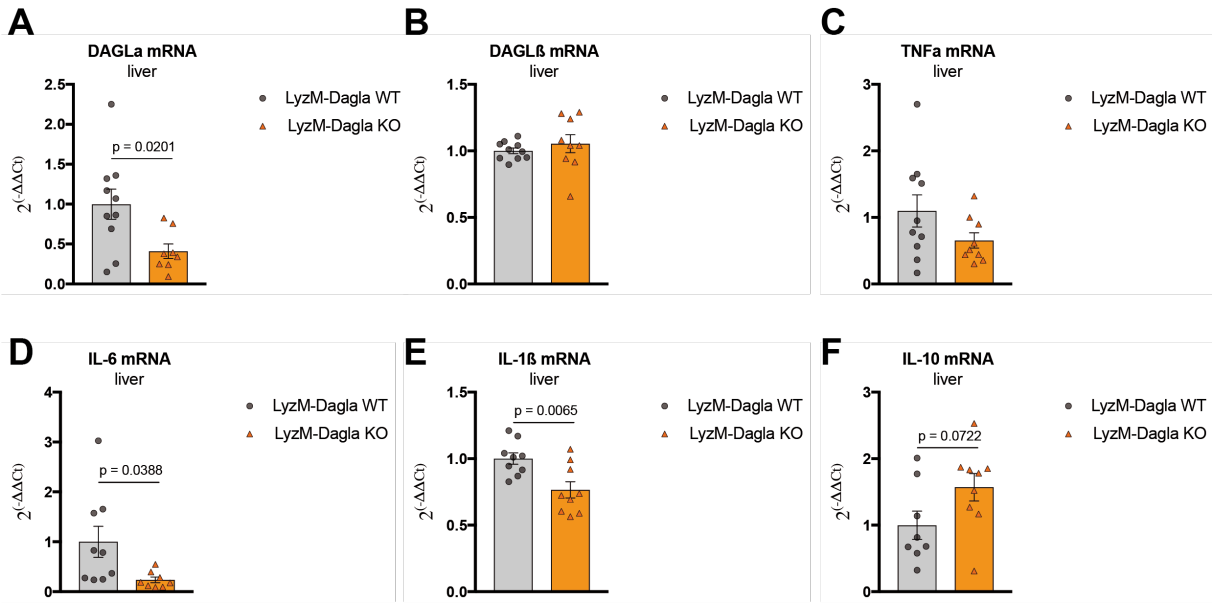


Figure 3.49 Expression analysis of different cytokines in the liver tissue of LyzM-Dagla KO mice.

(A) *DAGLa mRNA levels were reduced in liver of LyzM-Dagla KO mice compared to control mice.* ***(B)*** *Expression levels of DAGLb were unchanged.* ***(C)*** *mRNA levels of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFa) were unchanged in LyzM-Dagla KO liver.* ***(D,E)*** *Expression of both pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-1beta (IL1β) were significantly downregulated in the liver tissue of LyzM-Dagla KO mice compared to controls.* ***(F)*** *The mRNA levels of the anti-inflammatory cytokine interleukin 10 (IL-10) were increased in LyzM-Dagla KO mice. Values represent mean ± SEM; n = 8-10 animals/group. Students t-test.*

In summary, LyzM-Dagla KO mice show a lower inflammatory state (hypoinflammation) compared to control mice, opposing our hypothesis.

3.4 Sex differences in the endocannabinoid system

Possible sex differences in the endocannabinoid system were investigated in male in female Dagla fl/fl control mice using mRNA expression analysis and targeted lipidomics. The stage of the estrous cycle was controlled in female mice in this experiment. Only female mice in di- or metestrus were used since endocannabinoid levels are known to be changed during and previous to ovulation (Bradshaw et al. 2006). First, mRNA levels of proteins involved in the endocannabinoid system

were compared in the hippocampal tissue of male and female Dagla fl/fl control mice (Figure 3.50A). mRNA levels of *Dagla* and *Daglb*, were similar in the hippocampus of male and female Dagla fl/fl mice. There were no differences in the expression of the cannabinoid receptor *CB1* between male and female mice, whereas mRNA levels of the cannabinoid receptor CB2 were too low to be detected in the brain tissue of either of the genders. The enzymes degrading 2-AG (*MAGL, ABHD6, and ABHD12*) were similarly expressed in the hippocampi of male and female mice, as well as the enzyme degrading anandamide (*FAAH*). Brain-derived neurotrophic factor (*BDNF*) mRNA levels were also similar in male and female mice. Next, expression of the corresponding proteins was investigated in cortical tissue of male and female Dagla fl/fl control mice (Figure 3.50B). Here, a tendency for lower expression of *Dagla* ($p=0.08$) and *Daglb* ($p=0.06$) in the cortex of female mice was measured compared to that of males. CB1 was significantly less ($p=0.02$) expressed in the cortex of females. Again, *CB2* mRNA level in the cortex was too low to be detected. In cortex of female mice, *MAGL* was significantly lower expressed compared to males ($p=0.008$). The other two metabolizing enzymes of 2-AG, *ABHD6 and ABHD12*, were similarly expressed in male and female cortex. Messenger RNA levels *of FAAH and BDNF* were also similar in the cortex of male and female mice.

Next, lipid levels in different brain regions of male and female Dagla fl/fl control mice were measured by LC-MS/MS at the University of Bern. 2-AG levels were similar in the PFC, striatum, and hippocampus of male and female mice (Figure 3.50C). Anandamide levels were higher in PFC of female mice compared to males, but in the striatum and hippocampus the levels were similar (Figure 3.50D). Arachidonic acid as well as SAG levels were similar in all tested brain regions between male and female mice (Figure 3.50E,F).

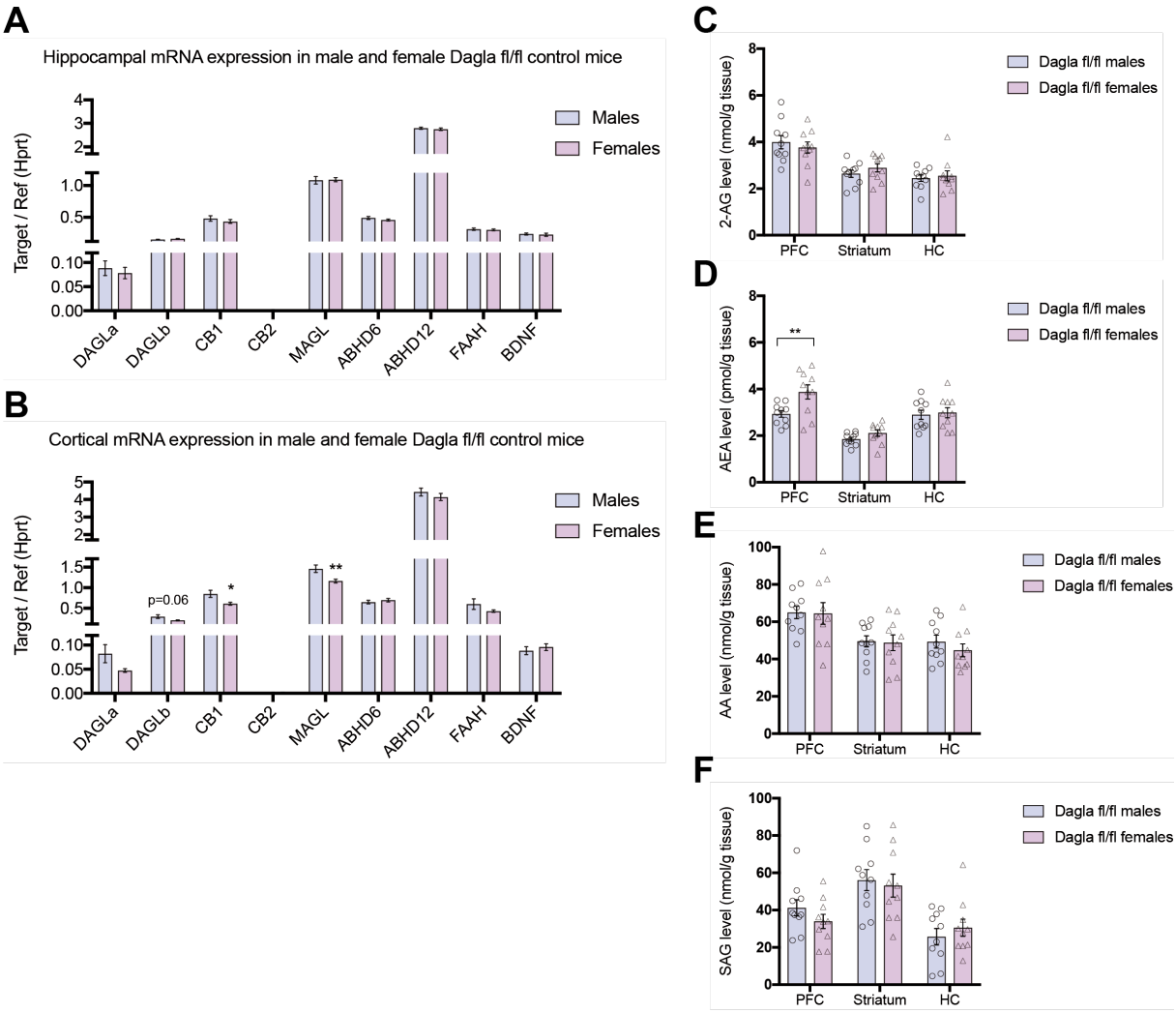


Figure 3.50 Sex differences in endocannabinoid system components measured by expression analysis (RT-PCR) and targeted lipidomics

(A) *In the hippocampus no differences in the expression of DAGLa, DAGLb, CB1, MAGL, ABHD&, ABHD12, FAAH or BDNF were detected between male and female Dagla fl/fl control mice. CB2 could not be detected because the expression in the brain is too low.* ***(B)*** *In cortex, the expression of DAGLa (p=0.08) and DAGLb (p=0.06) were slightly decreased in female mice compared to males. CB1 expression was significantly lower (p=0.02) in cortex of females, whereas CB2 could not be detected. The 2-AG metabolizing enzyme MAGL was lower expressed in females (p=0.008). ABHD6 and ABHD12 were similarly expressed in cortex of male and female Dagla fl/fl control mice. Expression of FAAH and BDNF was similar in cortex of male and female mice.* ***(C)*** *2-AG levels were similar between males and females in all tested brain regions.* ***(D)*** *AEA levels were higher in female mice in PFC compared to males (p=0.004). In all the other regions, levels were similar.* ***(E,F)*** *The levels of AA and SAG were similar in all tested brain regions between male and female mice. Values represent mean ± SEM; n=10 animals/group. Students t-test in RT-PCR; Two-way ANOVA and Sidak´s multiple comparisons for lipid measurements. Targeted lipidomics were performed by Sandra Glasmacher, University of Bern.*

Altogether, it seems that CB1 and MAGL are lower expressed in the cortex of female mice, whereas the expression in the hippocampus is similar. Nevertheless, the lipid levels were mostly similar between males and females, with the exception of anandamide levels that were higher in PFC of females.

4 Discussion

The endocannabinoid system is an important neuromodulatory system. Furthermore, it is known to play a key role in the regulation of emotional responses. Studies using genetic deletion of *Dagla* (main producing enzyme of the endocannabinoid 2-AG) have found profound changes in endocannabinoid levels and in affective behaviors. However, the cellular source of 2-AG was unknown. Thus, one aim of the study was to find the main producing cell type of 2-AG and to clarify the role of 2-AG production in different cell types in the regulation of affective behaviors. Therefore, conditional KO mice with a specific deletion of *Dagla* in neurons, astrocytes and microglia were used in this study. For characterization of these mouse lines, molecular analysis and the highly sensitive RNAScope technology were performed. In order to find the cellular source of 2-AG, targeted lipidomics in different brain regions of conditional Dagla KO mice were conducted. Moreover, mouse behaviors were assessed in different behavioral tests to investigate the role of cell-type specific 2-AG production in the regulation of affective disorders. A hallmark of affective disorders in mouse models are dysregulations of adult neurogenesis, a process that is, amongst others, regulated by endocannabinoids. Therefore, the last aim was to investigate adult neurogenesis in our conditional Dagla KO mice.

4.1 DAGLa in neurons

To investigate the role of DAGLa in neurons, conditional Syn-Dagla KO mice with Cre expressed under a synapsin (Syn)-promoter and their littermate controls (Dagla fl/fl) were used. Analysis of DAGLa expression in this mouse line by immunohistochemistry revealed a reduction of DAGLa protein in the dentate gyrus, CA3, thalamus and basolateral amygdala (BLA), but not in the CA1 region of hippocampus. Analyzing the Syn-RosaTomato reporter line, we confirmed that DAGLa was deleted specifically in neurons in this mouse line. However, the efficacy of the KO was varying considerably depending on the brain region. For example, in the dentate gyrus, 86.3% of all neurons were expressing Cre, while only 16.2% of all neurons in the hypothalamus were Cre-positive. Apparently, the Syn-promoter is only active in specific neuronal subtypes that remain to be determined in future studies. For instance in the hippocampus, Cre was strongly expressed in dentate gyrus granule neurons projecting to CA3 (mossy fibers), but was not present in CA1 pyramidal cells in Syn-RosaTomato reporter line. McLean *et al*. identified several neuronal subtypes, including tyrosine hydroxylase (TH)-positive dopaminergic cells in substantia nigra, striatal dopamine- and cAMP-regulated neuronal phospho-protein (DARPP-32)-positive neurons, and choline acetyltransferase (ChAT)-positive motor neurons with an active Syn-promoter, using an adeno-associated viral gene transfer of the construct with the green fluorescent protein (GFP) under

the control of Syn-promoter in neonatal mice (McLean et al. 2014). Such characterization is important for other studies investigating neuronal deletion of genes using Syn-Cre mice.

Even though neurons were always postulated to be the main producers of 2-AG, Syn-Dagla KO mice did not show any changes in bulk 2-AG levels in the brain compared to littermate Dagla fl/fl controls. One possible reason might be the low efficacy of the Syn-promoter, leading to changes in 2-AG levels below the range of detection. Another reason could be the interplay between neurons and astrocytes in the production of 2-AG, because only GFAP-Dagla KO line (deletion in both, astrocytes and some neurons) showed strong reductions in bulk 2-AG levels. Accordingly, astrocytes might compensate for the loss of DAGLa on neurons, whereas neurons might compensate for the loss of DAGLa on astrocytes in GLAST-CreERT2-Dagla KO mice. However, we were able to measure an increase of DAGLa substrate SAG and a decrease in the levels of 2-AG metabolites arachidonic acid (AA) and prostaglandins (PGs) in Syn-Dagla KO mice. 2-AG levels are generally low (2-10 nmol/g) compared to SAG or AA levels (20-200 nmol/g), so that astrocytes might be able to compensate for 2-AG levels but not for SAG or AA levels. Another explanation would be a compensatory downregulation of MAGL or an upregulation of DAGLb in Syn-Dagla KO brains. At least in the hippocampus of Syn-Dagla KO mice, however, the expression of DAGLb was not altered (Figure 3.15), suggesting that this is not the reason for unchanged 2-AG content. Furthermore, Daglb seems to have a more important role on microglia cells during inflammatory processes and not such an important role on neurons under normal conditions, according to a recent study (Viader et al. 2016). To further verify this, the expression of MAGL in Syn-Dagla KO mice should be determined as well. Moreover, 2-AG measurements should be performed in a brain region with high efficacy of the Syn-promoter, e.g. the CA3, to clarify if the low efficacy of Syn-promoter was a reason for not detecting changes in bulk 2-AG levels.

Next, we measured body weight and food intake of Syn-Dagla KO mice in order to investigate any changes in metabolic processes due to the neuronal KO of *Dagla*. Syn-Dagla KO mice showed a reduced body weight and food intake compared to Dagla fl/fl control littermates. A similar phenotype was observed in constitutive Dagla KO mice (Jenniches et al. 2016a), but not in any of the other conditional Dagla KO mice tested in this thesis (only a tendency was found in GFAP-Dagla KO mice), clearly suggesting that neuronal DAGLa is responsible for the regulation of food intake and body weight under normal baseline conditions. Furthermore, CB1-KO mice showed a similar reduction in food intake and body weight (Ravinet Trillou et al. 2004), showing that decreased 2-AG signaling rather than increased precursors or decreased metabolites of 2-AG are responsible for the observed phenotype. Several other studies support this hypothesis. For instance, the CB1 antagonist rimonabant has been used to treat obesity (Curioni and André 2006) and smoking marijuana is known to highly increase appetite (Carai et al. 2006). In general, the hypothalamus is known to be responsible for the regulation of body weight and food intake. CB1 signaling in the hypothalamus functions to maintain food consumption and is suppressed by the satiety signal leptin (Vincenzo Di Marzo et al. 2001). Also in hypothalamic nuclei that modulate energy

homeostasis via interactions with appetite-inducing peptide ghrelin, CB1 receptor is expressed (Kola et al. 2008). Decreased 2-AG production of neurons in our Syn-Dagla KO mice and associated decrease in CB1 signaling in the hypothalamus might disrupt all the before-mentioned mechanisms regulating food intake and energy homeostasis. However, studies using viral-mediated deletion of the CB1 receptor gene in the hypothalamus observed decreased body weight of the mice but normal food intake (Cardinal et al. 2012). The phenotype of reduced body weight and food intake was more pronounced in female Syn-Dagla KO mice, while both, body weight and food intake equaled out in males with aging. An explanation would be a possible interaction of sex-hormones with the endocannabinoid system. This would be also indicated by the fact that only female GLAST-CreERT2-Dagla KO mice showed a phenotype in behavioral tests. Moreover, female Syn-Dagla KO mice showed more drastic changes in CORT levels that could be associated with decreased body weight and food intake.

Despite lower body weight and food intake, Syn-Dagla KO mice showed normal survival. None of the mice died during the behavioral testing. Nevertheless, Syn-Dagla KO mice displayed a lower area covered with GFAP in the hilus, contrary to a bigger area covered with GFAP that was observed in GFAP-Dagla KO mice. The number of astrocytes was not altered in hilus, suggesting a change in morphology and GFAP expression in astrocytes of Syn-Dagla KO mice. Reduction in GFAP expression might be correlated with the decrease of PGD2 levels in Syn-Dagla KO brains. Studies have shown that astrocytes increase GFAP expression upon stimulation of their prostaglandin D2 receptor. Moreover, this study showed that a blockade of PGD2 signaling resulted in a remarkable suppression of astrogliosis and demyelination (Mohri et al. 2006). The question of why the changes in astrocytes are contrary in Syn-Dagla KO and GFAP-Dagla KO brains is hard to interpret and needs further investigation. One hypothesis is that highly increased CORT levels in Syn-Dagla KO mice (discussed later on) might reduce reactive gliosis because of its anti-inflammatory properties (Little et al. 1998).

In order to test for behavioral changes mediated by neuronal *Dagla* deletion, control and Syn-Dagla KO mice were tested in different paradigms assessing anxiety-related and depression-like behaviors. The results indicate changes in exploratory behavior and anxiety-related behaviors in mice lacking DAGLa in neurons. First of all, both males and female mice showed reduced locomotor activity in the home cage, especially during the dark/active phase. This behavior can be associated with decreased food intake and body weight - probably leading to decreased energy availability. Moreover, hypoactivity was also observed in CB1 KO mice (Zimmer et al. 1999) and the current result confirms the role of the endogenous cannabinoid system in the modulation of motor activity. In anxiety-related tests, male Syn-Dagla KO mice showed not a straight-forward phenotype. In the open-field test and o-maze only male mice spent more time in the open/central areas, which is actually associated with a behavior of decreased anxiety. However, in the social avoidance test, male Syn-Dagla KO mice showed increased social anxiety. In the light/dark test, Syn-Dagla KO mice again displayed “braver” behaviors compared to their littermate control mice, spending more time

in the illuminated area. These contrary effects on anxiety in different tests are in line with the biphasic effect of cannabinoids on anxiety that is often observed in the literature (Szuster, Pontius, and Campos 1988; Thomas 1996). Here, different doses of cannabinoids or activation of cannabinoid system in different brain regions led either to anxiolytic or anxiogenic effects (Lisboa et al. 2008; E. S. Onaivi et al. 1995; Patel and Hillard 2006; Rubino et al. 2008; Zanettini et al. 2011). However, the reasons for this biphasic effect of cannabinoids on anxiety are not fully elucidated. Previous studies investigating deletion of CB1 showed contradictory behavior in the dark/light test compared to our Syn-Dagla KO mice, leading to a decreased time spent in the light area (Martin et al. 2002). Thus, the effects observed in Syn-Dagla KO mice might be partly assigned to other changes in arachidonate lipidome. The behavioral response of Syn-Dagla KO mice in anxiety tests could also be explained by findings of a study using conditional CB1-KO mice with a deletion of *CB1* specifically on cortical glutamatergic and GABAergic neurons. In this study, it was found that deletion of *CB1* specifically on cortical glutamatergic terminals is important for the acquisition of active avoidance, whereas CB1 receptors on GABAergic neurons mediate the acquisition of passive avoidance, as the absence of CB1 on those neurons establishes a strong bias toward escape behavior, similar to the behavior that we observed in our Syn-Dagla KO mice (Genewsky and Wotjak 2017).

An interesting effect that we also observed in the anxiety tests is that male Syn-Dagla KO mice showed increased activity (measured by the distance moved) in the zero-maze and dark/light test, even though they were hypoactive under normal conditions in the home cage. Thus, we hypothesized that the observed behavior might be rather a panic-like phenotype than decreased anxiety and mice might have been trying to escape from the maze due to the activation of the flight-and-fight response. Responses to acute and chronic stress are predominantly manifested by the activation of the HPA-axis, leading to increased stress hormone levels. In rodents, the body's main stress hormone is the glucocorticoid corticosterone (CORT). To prove this hypothesis, we measured CORT levels in Syn-Dagla KO mice at the end of the experiment after decapitation and found extremely enhanced CORT levels in the brains of both male and female Syn-Dagla KO mice (LS-MS/MS). CORT in the brain mirrors the plasma CORT levels since CORT is readily diffusing in all tissues. In contrast to brain and plasma CORT levels, fecal corticosterone levels enable the analysis of long-term effects. In CORT measurements from feces by ELISA, which represents a 24 h mean of the daily CORT fluctuations, this increase was not observed, suggesting that the previously observed effect is not long-lasting, or was masked either by the floor effect or alterations in circadian fluctuation of CORT in KO mice.

Since all other conditional mouse lines did not show changes in CORT, this data further suggests that especially DAGLa on neurons is responsible for the regulation of hypothalamic–pituitary–adrenal (HPA)-axis. From literature, it is known that 2-AG plays a crucial role in stress responses. For instance, it was shown that 2-AG levels are rising in prefrontal cortex (PFC), hippocampus, and hypothalamus (but not in the amygdala) after psychological stressors, response to which is

considered to be CORT dependent (Evanson et al. 2010; Matthew N. Hill et al. 2011; M. Wang et al. 2012). Similarly, CORT application to PFC or hippocampus initiated a 2-AG increase (Matthew N. Hill et al. 2011; M. Wang et al. 2012), suggesting that 2-AG contributes to the termination of stress response. CB1 signaling, in general, can both inhibit and potentiate the activation of HPA-axis by stress; it was shown for example that loss of CB1 signaling (pharmacological or genetic deletion) can also result in increased CORT release in response to an acute stress (Cota et al. 2007; Matthew N. Hill et al. 2011; Patel et al. 2004; Wade, Degroot, and Nomikos 2006), similar to what was found in our Syn-Dagla KO mice. Our results indicate that 2-AG produced by neurons is highly critical in the termination of stress response and that loss of neuronal DAGLa leads to highly elevated CORT levels after low stress.

These elevated CORT levels are in line with the behavioral output observed in anxiety-related tests. Due to increased CORT (or possibly flight and fight response) Syn-Dagla KO mice show increased activity in those paradigms, even though they are hypoactive in situations without stress (home cage conditions). Furthermore, not only CORT levels determine the HPA-axis function but also, for instance, the expression of glucocorticoid receptors (GR) that mediate the majority of negative feedback mechanisms (Herman 1993). At least in the hippocampus, we did not observe changes in GR transcript levels in Syn-Dagla KO mice (Figure 3.15). Further experiments should be done to clarify if the negative feedback mechanism is impaired in the hypothalamus, measuring CRH and GR expression profile and the physiological function of HPA-axis. It is noteworthy that anxiety-related behaviors are mainly regulated by the amygdala and latest research has found that endocannabinoid signaling is a key player in the suppression of anxiety induced by amygdala-prefrontal circuitry (Marcus et al. 2019). Moreover, the RosaTomato reporter line crossed into the Syn-Cre showed particularly high Cre expression in the BLA, suggesting a high KO efficacy of *Dagla* especially in this region. Thus, decreased production of 2-AG by neurons in this area might decrease the inhibition of this circuitry leading to increased anxiety/panic in Syn-DAGLa KO mice (or at least to changed behavior in anxiety-related tests).

Syn-Dagla KO mice did not show depression-like behavior in the nestlet test or anhedonia in the sucrose preference test, implying that neuronal DAGLa is not involved in the regulation of depression-like behavior seen in constitutive Dagla KO mice (Jenniches et al. 2016a). In the forced swim test, female, but not male Syn-Dagla KO mice showed an increased immobility time, which is a sign of depression-like behavior. However, since female Syn-Dagla KO mice also showed highly reduced body weight and food intake before the test, increased immobility time in the forced swim test might be affected by a lack of energy. For male Syn-Dagla KO mice the body weight and food intake phenotypes were not present at the time point of forced swim test anymore. To verify if increased immobility time in female mice could have been induced by a lack of endurance, we measured immobility time in the first two min (data not shown) of the test (normally only last four min of six min testing period are quantified). In the first two min of the test, female Syn-Dagla KO mice did not show a significantly increased immobility time, suggesting that the observed

phenotype is indeed initiated by a lack of energy. According to the literature, body weight can influence the behavior in the forced swim test (Bogdanova et al. 2013). Our data implies that depression-like phenotype observed in CB1/Dagla KO mice (Jenniches et al. 2016a; Valverde and Torrens 2012a), is mediated by loss of DAGLa in another cell type than neurons.

Maternal care behavior, assessed in the pup retrieval test, was unchanged in Syn-Dagla KO dams. Conspicuously, Syn-Dagla KO dams showed increased mortality, even though survival of female Syn-Dagla KO mice not used for breeding seemed to be normal. The reason for increased mortality might be again the leaner phenotype of this line since pregnancy is very energy-consuming. Further investigations need to be done to find the exact reasons.

Adult neurogenesis was unaltered in Syn-Dagla KO mice, even though mice showed a high deletion efficacy of *Dagla* in the dentate gyrus region. We could not detect any changes in proliferation or survival, measured by BrdU staining, neither did we find any changes in the differentiation of neural progenitor cells (NPCs). This result was unexpected since cannabinoids are known to promote cell proliferation in the dentate gyrus (Aguado et al. 2005b) and constitutive KO mice showed decreased progenitor cell proliferation (Y. Gao et al. 2010; Jenniches et al. 2016a). Apparently, the promotion of adult neurogenesis by endocannabinoids is implemented by another cell type than neurons of the dentate gyrus. To investigate if the expression of proteins involved in the regulation of adult neurogenesis was changed in Syn-Dagla KO mice, we performed a transcription analysis by RT-PCR. Most of the mRNA levels of the genes tested, were similar in Syn-Dagla KO mice and Dagla fl/fl control mice. Only the expression of brain-derived neurotrophic factor (BDNF) was increased in Syn-Dagla KO mice, which was unexpected again because CB1 KO mice are actually known to show reduced BDNF levels (Aso et al. 2008). Several studies have provided molecular and functional evidence for a crosstalk between BDNF and endocannabinoid signaling which is not fully understood yet (Maison et al. 2009; L. Zhao, Yeh, and Levine 2015). Cannabinoids have been shown to induce CREB phosphorylation (Isokawa 2009) and to promote changes in BDNF and CREB gene expression (Grigorenko et al. 2002). Both, cannabinoid signaling and BDNF are known to promote cell proliferation and act in synergism (De Chiara et al. 2010; Galve-Roperh et al. 2013). Interestingly, the increase in the subventricular zone (SVZ) and dentate gyrus pool of stem/progenitor cells mediated by BDNF was fully abolished in the presence of a CB2 antagonist but not CB1 antagonist, suggesting a more prominent role in CB2 signaling in the BDNF-induced increase of proliferation (Ferreira et al. 2018). It was also shown that BDNF can influence neuronal sensitivity to endocannabinoids in cultured granule neurons. Here, BDNF increased the expression of CB1 receptor transcripts and decreased the expression of MAGL transcripts (Maison et al. 2009). However, in our study, no upregulation of CB1 transcripts was observed in the hippocampus of Syn-Dagla KO mice. It might be possible that loss of 2-AG production in neurons increases BDNF production as a compensatory mechanism to rescue adult neurogenesis (Figure 4.1). A similar effect was seen in a study were increased levels of BDNF were shown to rescue the cognitive deficits promoted by Δ9-THC administration (Segal-Gavish et al. 2017). Recently it has been shown

that BDNF can also induce endocannabinoid release in the neocortex (Yeh, Selvam, and Levine 2017). Thus, increased BDNF expression might be a compensatory process to stimulate endocannabinoid release upon decreased availability of 2-AG produced by neurons. BDNF binds to the tropomyosin receptor kinase B (TrkB), a typical tyrosine kinase receptor that transduces the BDNF signal via Ras-ERK, PI3K, and activation of PLC. Activation of PLC increases the production of DAG (also seen in our lipid measurements) but there is no DAGLa on neurons in Syn-Dagla dentate gyrus to convert DAG to 2-AG. In general, BDNF expression should be further analyzed on protein level because there could also be a translational problem of BDNF synthesis.

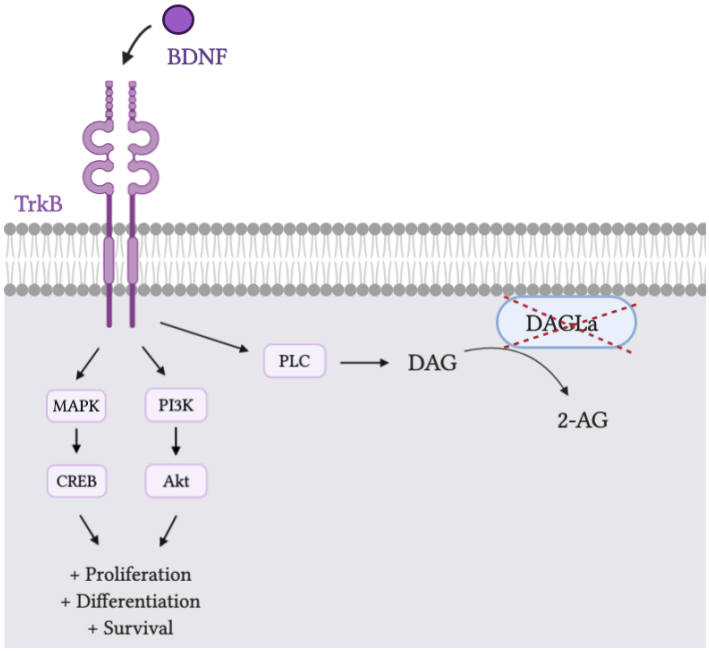


Figure 4.1: Possible compensatory mechanism for decreased proliferation in Syn-Dagla KO dentate gyrus by brain-derived neurotrophic factor (BDNF) signaling.

BDNF, which is overexpressed in hippocampus of Syn-Dagla KO mice, binds to tropomyosin receptor kinase B (TrkB), leading to phosphorylation of the receptor on different sites. Upon phosphorylation, downstream signaling pathways like MAPK/CREB or PI3K/Akt are activated causing an increase of proliferation, differentiation and survival that might compensate for any problems in adult neurogenesis in the hippocampus of Syn-Dagla KO mice. Furthermore, phosphorylation of TrkB activates the phospholipase C pathway leading to an increased production of diacylglycerol (DAG), the substrate of DAGL. This might also be a compensatory mechanism to normalize 2-AG levels, but DAGLa is missing to convert DAG to 2-AG.

Since proper cannabinoid signaling is essential for normal brain development and Syn promoter is active from embryonic day 12 (Melloni and Degennaro 1994), it is difficult to tell if all the observed molecular changes and changes in behavior are a direct consequence of disrupted 2-AG biosynthesis or rather an indirect consequence of developmental effects. The usage of an inducible Cre recombination would be helpful to study this.

To conclude, our data suggest an important role of DAGLa in neurons, especially in the regulation of body weight, food intake, and stress/anxiety. In the regulation of stress, neuronal DAGLa has a key role in negative feedback mechanisms to the HPA-axis, this is supported by the highly upregulated CORT levels observed in Syn-Dagla KO mice after mild stress in the current study.

Neuronal DAGLa is clearly involved in the production of 2-AG, measured by decreased metabolites and increased precursors of 2-AG. However, it seems like either astrocytic DAGLa is able to compensate 2-AG levels due to the loss of DAGLa in neurons or our Cre-line was not efficient enough to induce changes in bulk 2-AG levels. Last, lack of neuronal DAGLa seems to have effects on BDNF expression in the hippocampus in the opposite way compared to the deletion of CB1.

4.2 DAGLa in astrocytes

Until now it has not been shown whether DAGLa is actually expressed in astrocytes of the adult mouse brain. Therefore, we first investigated the expression of DAGLa in Dagla fl/fl control mice. Using a highly sensitive and specific *in situ* expression analysis technology (RNAscope), we showed for the first time that *Dagla* is expressed in astrocytes of the adult mouse brain. Previously astrocytic DAGLa expression has only been shown in cell culture and on spinal cord astrocytes (Hegyi et al. 2018; Oudin, Hobbs, and Doherty 2011; Rodriguez, Mackie, and Pickel 2001; Viader et al. 2015). This finding further highlights the importance of endocannabinoid signaling in astrocytes, which has only recently been recognized. The vast majority of the endocannabinoid receptor CB1 is localized in neurons (Oliveira da Cruz et al. 2016). Nevertheless, CB1 is clearly expressed in astrocytes *in situ* (Rodriguez, Mackie, and Pickel 2001) and despite its low expression levels, the receptor modulates critical aspects of neuron-astroglial interactions, as shown by a number of physiological and genetic studies (Han et al. 2012b; M. Navarrete, Diez, and Araque 2014; Marta Navarrete and Araque 2008; Robin et al. 2018; Walter and Stella 2003). More specifically, the activation of astrocytic CB1 receptors triggers Ca^{2+} transients in astrocytes, stimulates the release of gliotransmitters and regulates carbohydrate and lipid metabolism. Interestingly, studies investigating the localization of CB1 and DAGLa in astrocytes of the spinal cord and the developing mouse brain indicate that both proteins are localized in close proximity (Hegyi et al. 2018; Oudin, Hobbs, and Doherty 2011; Rodriguez, Mackie, and Pickel 2001).

We showed that approximately 40-70% of the astrocytes positive for the pan-astrocytic marker *Aldh1l1* express *Dagla*. On the other hand, *Dagla* mRNA was detected in almost all neurons analyzed. This indicates that *Dagla* expression is restricted to a sub-population of astrocytes, but it is currently unclear what the cellular characteristics of this sub-population are. Similar to CB1 receptors, the expression level of *Dagla* in the mouse brain astrocytes was low, only about 10% of that of neurons. mRNA signals were often observed outside the cell body, indicating that DAGLa may belong to proteins that are locally translated in distal processes, like it has been shown for other astrocytic proteins (Boulay et al. 2017; Sakers et al. 2017). It is thus possible that we have underestimated the amount of *Dagla* transcribed in astrocytes since the fine astrocytic processes could not be identified.

To address the significance of astrocytic 2-AG production and associated arachidonate remodeling, we generated conditional KO mice lacking DAGLa in astrocytes: GFAP-Dagla and inducible GLAST-CreERT2-Dagla KO mice. Our RNAscope results showed that the deletion of *Dagla* in the GLAST-CreERT2-Dagla KO line was specific to astrocytes, affecting up to 50% of this cell population. This was confirmed with tdTomato reporter mice, which also showed that 37-79% of S100b positive astrocytes express Cre, with no expression in neurons. The RNAscope analysis of GFAP-Dagla KO mice, on the other hand, showed a much higher deletion of *Dagla* in astrocytes (up to 93% in the cingulate cortex), but also an off-target deletion of *Dagla* in neurons in some brain regions. The off-target effect was probably due to an activity of the GFAP promoter in neuronal stem cells (NSCs) during brain development (Götz and Barde 2005). This finding is relevant for interpreting other studies using GFAP-Cre mice to address gene functions in astrocytes.

Subsequently, we measured the levels of lipids involved in the DAGLa cascade from different brain regions in our astrocytic Dagla KO mice to assess the impact of astrocytic DAGLa on 2-AG production and connected arachidonate remodeling. In female, but not male GLAST-CreERT2-Dagla KO animals, a slight reduction in 2-AG and arachidonic acid (AA) levels was detected. Considering the fact that astrocytes are approximately as numerous as neurons in the brain, these results strongly suggest that the contribution of astrocytes to steady-state brain 2-AG and AA levels is relatively small compared with that of neurons. This finding is in good agreement with the modest expression levels of *Dagla* mRNA in astrocytes in comparison to neurons. Nevertheless, Syn-Dagla KO mice also did not show significant alterations in bulk 2-AG levels. It is thus possible that neuronal DAGLa can compensate for the loss of DAGLa on astrocytes by increasing its activity and 2-AG production and vice versa. Since GLAST-CreERT2 only affects up to 50% of astrocytes it is further possible that we underestimate the contribution of astrocytic DAGLa in the production of 2-AG. In contrast, GFAP-Dagla KO mice showed an approximately 65% reduction of 2-AG levels in PFC and 70% in the hippocampus. The strongly reduced 2-AG levels highly likely reflect the neuronal off-target deletion of *Dagla* in these regions. Concomitantly, SAG levels were increased and metabolic products of 2-AG, AA and decreased prostaglandins (Nomura et al. 2011b; Reisenberg et al. 2012), similar to the lipid profile seen in constitutive Dagla KO mice (Jenniches et al. 2016b; Schurman et al. 2019). The opposite effect can be observed after deleting the metabolic enzyme of 2-AG, MAGL (Viader et al. 2015). Interestingly, the levels of the endocannabinoid AEA were also significantly decreased in the hippocampus (and striatum in female mice) of GFAP-Dagla KO mice, like it was previously observed in constitutive Dagla KO mice (Y. Gao et al. 2010). It seems like a strong reduction of 2-AG production in GFAP-Dagla KO mice leads to a decrease in AEA synthesis or an increase in AEA degradation through an indirect and unknown mechanism. Not even the deletion of NAPE-PLD, the main producing enzyme of AEA, lead to a decrease in AEA levels (Leung et al. 2006), suggesting an alternative way in AEA production that seems to be affected in our mouse line.

Neither GFAP- nor GLAST-CreERT2-Dagla KO mice showed remarkable changes in body weight or food intake. Only male GFAP-Dagla KO mice showed a tendency towards a reduced body weight, showing that neuronal DAGLa alone (in areas other than the frontal cortex and the hippocampus) seems to be important in the modulation of these metabolic processes (see 4.1.). Interestingly, GFAP-Dagla KO mice showed a highly increased mortality (20%) rate which was also observed in CB1 KO mice (Zimmer et al. 1999). Furthermore, GFAP-Dagla KO mice were often suffering from epileptic seizures, which might be also one cause of death. GFAP-Dagla KO mice showed a highly increased area covered with GFAP in the dentate gyrus, while the number of astrocytes was unchanged. These results indicate that astrocytes in the dentate gyrus of GFAP-Dagla KO mice have changed their morphology - a typical sign of mild to moderate astrogliosis (Sofroniew 2015). Astrogliosis mainly occurs in response to CNS damage or disease, like traumatic or ischemic injuries or inflammation (Sofroniew 2015). It is possible, that astrogliosis is a consequence of the epileptic seizures of GFAP-Dagla KO mice since brain pathologies often induce astrocytes to become reactive. However, a study has shown that reactive astrocytes can also cause epileptic seizures, rather than the other way around, since inducing astrogliosis in a mouse model without pathologies lead to seizures (Robel et al. 2015). Another possibility is that the loss of 2-AG, which has anti-inflammatory potential (Patsenker et al. 2015), leads to partial inflammation in the brain that causes astrogliosis. The development of astrogliosis might be responsible for the seizures of GFAP-Dagla KO mice that might be also a reason for increased mortality in this mouse line. This hypothesis is confirmed by the fact that astrogliosis only occurs in brain regions of GFAP-Dagla KO mice in which both astrocytes and neurons are affected by the KO (dentate gyrus and the retrosplenial cortex), but not in the amygdala where only astrocytes are affected. Furthermore, astrogliosis was only found in the brains of GFAP-Dagla KO mice, which is the only line showing strong reductions in 2-AG levels. GLAST-CreERT2-Dagla KO mice showed a similar tendency but no significant changes in astrocyte morphology. Moreover, they did not suffer from epileptic seizures and did not show increased mortality.

Changes in astrocytes also have recently been proposed to be important in the etiology of depression (Q. Wang et al. 2017). Thus, we focused on the analysis of affective behaviors in astrocytic Dagla KO mice. Based on the targeted lipidomics data and the different *Dagla* KO penetrance in GFAP-Dagla KO (astrocytes and neurons) versus GLAST-CreERT2-Dagla KO (subset of astrocytes), we expected distinct phenotypes in these two lines, with a generally stronger effect of the *Dagla* deletion in GFAP-Dagla KO mice. Indeed, GFAP-Dagla KO mice showed changes in anxiety-related behavior similar to what was seen in constitutive Dagla KO mice (Jenniches et al. 2016b; Shonesy et al. 2014b). In contrast, we did not observe such a phenotype in GLAST-CreERT2-Dagla KO mice, indicating that the anxiety phenotype was produced by neuronal rather than astroglial *Dagla* deletion. Since Syn-Dagla KO mice also showed similar disturbances in exploratory and anxiety-like behaviors, this phenotype can be clearly assigned to the neuronal deletion of

Dagla. This finding also indicates that the deletion of *Dagla* from astrocytes did not cause a general disturbance in brain functions but rather affected specific mouse behaviors.

Conversely, both of the astrocytic Dagla KO lines showed alterations in tests for depressive-like behavior. Female GLAST-CreERT2-Dagla KO, with a deletion specific to astrocytes, and GFAP-Dagla KO mice from both sexes, displayed reduced nest-building behavior, anhedonia in the sucrose preference test and enhanced immobility in the forced swim test. Endocannabinoid signaling, especially via CB1, is known to modulate depression-like behavior in rodents and humans. CB1 receptor antagonist rimonabant increased depressive symptoms in humans. Moreover, increasing 2-AG levels by blocking MAGL produced antidepressant effects and increased adult hippocampal neurogenesis (Z. Zhang et al. 2015; Zhong et al. 2014). Correspondingly, the chronic treatment with antidepressants is known to significantly increase 2-AG levels in various brain areas, indicating that 2-AG could be important for the antidepressant effect of these drugs (Smaga et al. 2014). Our results now indicate that loss of 2-AG production by astrocytes leads to depressive-like behavior.

Most strikingly, GLAST-CreERT2-Dagla KO mice showed a profound deficit in the pup retrieval test compared to tamoxifen-injected Dagla fl/fl control dams. GLAST-CreERT2-Dagla KO dams needed significantly more time to retrieve their pups back to the test, or (in most cases) they did not retrieve the pups at all. However, most of the pups showed milk spots, suggesting that nursing was unaffected by the deletion of *Dagla* on astrocytes. This phenotype was previously observed in complete Dagla KO mice (Jenniches et al. 2016b) and is also present in GFAP-Dagla KO mice. Furthermore, a similar phenotype has been observed in dams treated with the CB1 receptor antagonist rimonabant (Schechter 2012). In contrast, Syn-Dagla mice, with a *Dagla* deletion in neurons, retrieved their pups normally. Thus, the maternal care phenotype can be clearly attributed to the lack of *Dagla* in astrocytes.

It should be noted that all mice used in behavioral tests, other than that assessing maternal behavior, were derived from paternal breedings, wherein both *Dagla* alleles were functional in dams. This breeding scheme excluded the possibility that the maternal care phenotype interfered with the anxiety or depressive-like behavior of the offspring. Furthermore, mice with GLAST-Cre insertion, without tamoxifen injections, showed normal pup retrieval as well (data not shown), showing that this phenotype was not created by a heterozygous KO of the GLAST transporter. On the molecular level, female GFAP-Dagla KO mice did not display changes in oxytocin, vasopressin or Crh expression in the hypothalamus that could have affected maternal care behavior. Further analysis should be done in GFAP-Dagla KO or GLAST-CreERT2-Dagla KO dams since motherhood could change the expression of those hormones or their release leading to the observed changes.

Vision and olfaction were unaffected by the deletion of *Dagla* in astrocytes, confirming that maternal care deficits were not caused by impairments of these senses. Interestingly, GFAP-Dagla KO mice displayed less time spent with either of the cotton swabs in the olfaction test, suggesting that these mice, in general, have less interest in novel things or decreased motivation. Similarly, cannabinoid receptor stimulation was found to affect novelty preference in rats (Fox, Sterling, and

Van Bockstaele 2009). It is important to note that motivational dysfunctions are also shared among affective disorders, including depression (Russo and Nestler 2013). The motivational dysfunction thus also fits the depression-like phenotype observed in this study.

The observed maternal care deficits and depression-like behavior also fit into the framework of postpartum depression (PPD). Although the nosology of PPD is controversial and there is debate about whether it is a separate disorder from major depressive disorder (MDD), it is clear that depressive episodes during reproductive years are twice as common in women than in men. This difference is possibly caused by hormonal fluctuations in women that are particularly evident in the peripartum period (Di Florio and Meltzer-Brody 2015; McEvoy et al. 2017a) and is supported by our observation of a stronger depressive-like phenotype in female mice. The etiology of PPD remains poorly understood, and novel animal models could help in elucidating the underpinnings of this complex disorder (Perani and Slattery 2014). Thus, GLAST-CreERT2-Dagla and GFAP-Dagla KO mice might be useful for studying PPD, particularly given that GFAP-Dagla KO females also display the anxiety-like behavior, an additional symptom of PPD (McEvoy et al. 2017b).

It is also noteworthy that only female GLAST-CreERT2-Dagla KO mice displayed a slight decrease of 2-AG levels and depressive-like behavior, indicating sex differences in the amount and effects of 2-AG produced by astrocytes. Sex differences in the occurrence of depression-related disorders have been well documented in humans, wherein the prevalence of depression is known to be significantly higher in women than that in men (Lim et al. 2018). Thus, it is conceivable that sex-related differences in the endocannabinoid system contribute to the etiology of depression, in addition to other endocrine differences. Another explanation for the sex differences observed in depression-like behaviors could be an abdominal swelling in male GLAST-CreERT2-Dagla KO and Dagla fl/fl control mice after tamoxifen injections, that may have influenced behavioral results in these animals. It has been shown that tamoxifen increased collagenase activity resulting in hernia in male mice only (X. Ma et al. 2015). When comparing the performance of male tamoxifen-injected Dagla fl/fl mice (GLAST-CreERT2-Dagla KO littermate controls) with male Dagla fl/fl that were not injected with tamoxifen (GFAP-Dagla KO littermate controls), we found that male tamoxifen-injected Dagla fl/fl mice with swollen abdomen/hernia showed significantly decreased distance moved in open-field, higher immobility time in the forced swim test and a lower sucrose preference compared to uninjected control mice.

Although we cannot exclude the possibility that the behavioral phenotypes characterized in this thesis are due to changes in the arachidonate lipidome, it seems reasonable to postulate that they are primarily caused by impaired production of 2-AG. Thus most of the phenotypes observed in this study, after *Dagla* deletion in astrocytes, were also observed in CB1 KO mice (Haller et al. 2002; Poncelet et al. 2003; Valverde and Torrens 2012b). Furthermore, the pharmacological blockade of CB1 by rimonabant in dams had the same effect on maternal care (Schechter et al. 2012) and pharmacological blockade of CB1 receptors increased the occurrence of depressive symptoms in humans (Sam, Salem, and Ghatei 2011). Therefore, behavioral changes observed in astroglial

Dagla KO mice could have resulted from the disruption of CB1 receptor activation by the lack of 2-AG. It is interesting to note that reduced basal 2-AG levels have also been demonstrated in patients with depression and post-traumatic stress disorder (Matthew N. Hill et al. 2013a; Matthew N Hill et al. 2009; Hillard and Liu 2014), which is consistent with the possible involvement of 2-AG in these disorders. On the other hand, non-targeted lipidomics in patients with MDD have also shown changes in the DAGLa substrate diacylglycerol (Liu et al. 2016). Furthermore, depression in humans is associated with decreased levels of the arachidonate metabolite prostaglandin D2 (PGD2). This finding in humans was substantiated by the inhibition of PGD2 biosynthesis in mice, which also produced depression-like behavior (Chu et al. 2017). Therefore, changes in the lipid signaling network from DAGLa deletion probably converge to influence depressive-like and maternal behaviors.

An alternative possible mechanism for the affective phenotype in astrocyte-specific KO lines could be a deletion of *Dagla* in NPCs. According to analysis of RNAscope data and RosaTomato reporter line, the KO of *Dagla* in GLAST-CreERT2-Dagla KO mice does not affect many astrocytes in the dentate gyrus. In fact *Dagla* is especially knocked out in NSCs and NPCs in the dentate gyrus in this mouse line, which led to the supposition that autocrine endocannabinoid signaling in those cells is promoting proliferation in the dentate gyrus. Due to the deletion of DAGL in NPCs in both lines, there is a lack of 2-AG production and a concomitant lack of autocrine activation of endocannabinoid receptors that might lead to decreased proliferation of the cells. On the other hand, impairments in adult neurogenesis per se are a hallmark of depression in animal models. Both, GLAST-CreERT2- and GFAP-Dagla KO mice showed decreased proliferation of NPCs in the dentate gyrus. Based on the data from previous studies one could hypothesize that the reduced neurogenesis resulting from the deletion of *Dagla* modulates affective behaviors (Y. Gao et al. 2010; Jenniches et al. 2016b; Medina and Workman 2018; B. R. Miller and Hen 2015). All available astrocyte-specific promoters, including the GLAST, GFAP and Aldh1l1 promoters, are active in NPCs of adult mice (Foo and Dougherty 2013; Mori et al. 2006b). To exclude this alternative hypothesis an inducible Cre-deleter strain that is exclusively active in NPCs would be required. To the best of our knowledge, such a mouse strain is currently not available.

CB1 KO mice, as well as constitutive Dagla KO mice, showed a similar decrease in adult neurogenesis (Jenniches et al. 2016a; Jin et al. 2004), but it is unknown yet, which cell type mediates this mechanism. NSCs contain a functional endocannabinoid system, including the CB1. A recent study has now shown, that the deletion of CB1 specifically on NSCs (Nestin-CreERT2-CB1 KO) leads to a similar effect of decreased proliferation in the hippocampus with concomitant depression-like phenotype as we observed in our astroglia-specific Dagla KO mice (Zimmermann et al. 2018). Since Syn-Dagla KO mice and microglia-specific Dagla KO mice did not show decreased proliferation of NSCs/NPCs in the dentate gyrus, it seems like either astrocytes or NSCs themselves produce 2-AG to promote proliferation. Other studies have already proposed 2-AG signaling to be functional in NSCs in an autocrine manner (Oudin, Hobbs, and Doherty 2011). In

both astrocytic lines, we observed a concomitant decrease of fibroblast-growth factor 2 (FGF2) in the hippocampus. FGF2 is known to be a growth factor important to maintain normal adult neurogenesis (Temple and Qian 1995). In neurogenic niches, FGF2 is produced by astrocytes, suggesting that astrocytes orchestrate proliferation by providing FGF2 to the NSCs (Newman, Féron, and Mackay-Sim 2000). Additionally, it was shown that DAGL activity is required for FGF-stimulated calcium influx into neuronal growth cones, and this response is both necessary and sufficient for an axonal growth response, suggesting a connection between DAGL and FGF2 signaling (Williams, Walsh, and Doherty 2003). A connection between DAGLa activity in astrocytes and FGF2 expression is a novel finding that requires further investigation, that is, however, beyond the scope of this thesis. Interestingly, it was also observed that treatment with tricyclic antidepressants significantly increased the expression of FGF2 in astrocyte cultures, suggesting a possible connection between the downregulation of FGF2 and the depression-like behavior in our mouse lines (Kajitani et al. 2012).

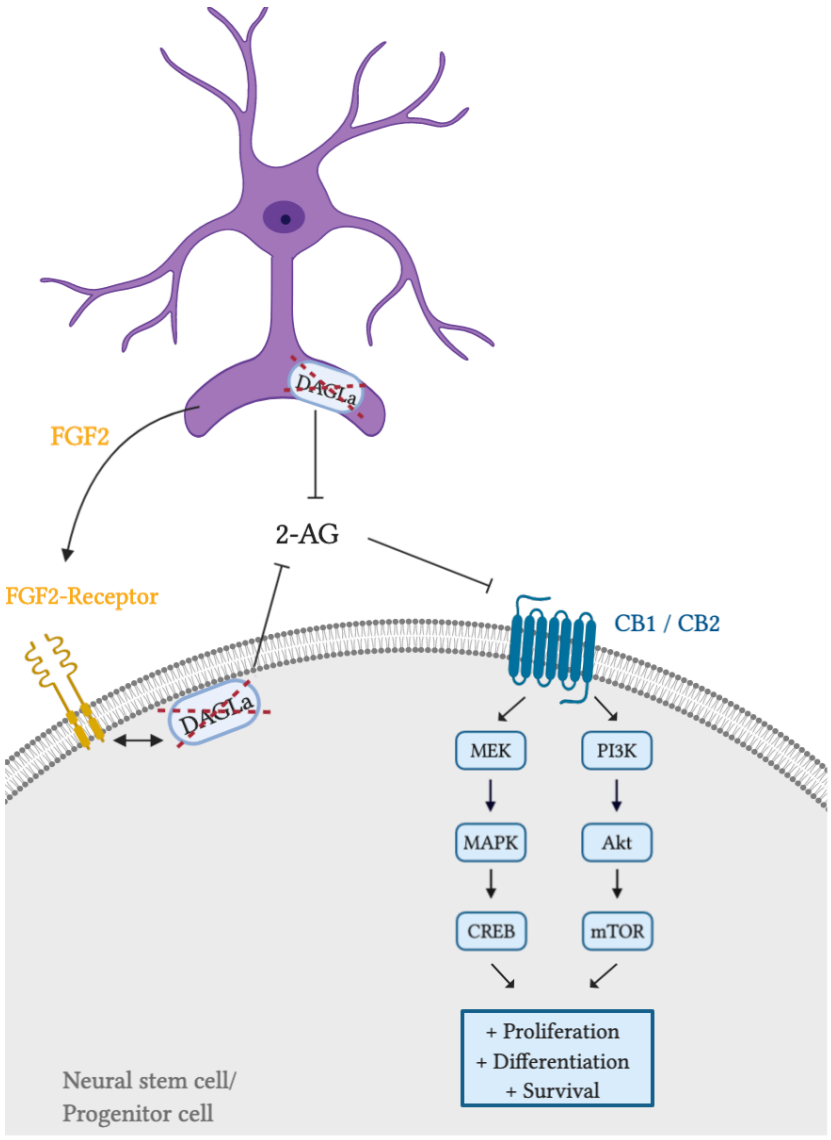


Figure 4.2: Possible mechanisms for reduced proliferation of neural stem cells or progenitor cells in dentate gyrus of GFAP-Dagla KO and GLAST-CreERT2-Dagla KO mice.

The deletion of Dagla in neural stem cells (NSCs) and progenitor cells (NPCs, in gray) might decreases 2-AG production of those cells, leading to reduced autocrine endocannabinoid signaling. Also, the deletion of Dagla in astrocytes (in purple, top of the picture) might reduce available 2-AG close to the NSC/NPC. Normally endocannabinoid signaling in NSCs/NPCs promotes proliferation, differentiation and survival of NPCs through MEK/MAPK/CREB or PI2K/Akt/mTOR signaling that is reduced in the KO lines. Furthermore, a connection between DAGLa activity and FGF2 signaling was shown in this thesis. It might be that KO of Dagla in astrocytes leads to decreased expression of FGF2, a growth factor that is also known to promote adult neurogenesis. This is a novel finding and needs further investigation.

To summarize this work, our data demonstrate that *Dagla* is expressed in a subpopulation of astrocytes and that *Dagla* expression in astrocytes is generally lower than in neurons. Although astrocytic DAGLa only accounts for a minor fraction of overall brain 2-AG levels and specific deletion of *Dagla* in astrocytes leads to small changes in the arachidonate lipid network, it nevertheless constitutes a hitherto unknown metabolic axis in the endocannabinoid system. Our data strongly suggest that minor and cell-specific changes in the extended endocannabinoid system can lead to strong behavioral effects, contributing to the understanding of psychiatric disorders such as

depression. Depression-like behavior in astrocytic Dagla KO mice is correlated with decreased adult neurogenesis that might be associated with changes in FGF2 expression.

4.3 DAGLa in microglia and myeloid cells

Two different Cre-driver lines were selected to target DAGLa in microglia, namely LyzM-Dagla and Cx3Cr1-Dagla KO lines. However, upon closer examination we discovered that Cx3Cr1-Dagla KO mice are suitable for the investigation of microglial cells, whereas LyzM-Dagla KO affects mostly peripheral myeloid cells but also some neurons. First, RosaTomato reporter line uncovered that LyzM-promoter is actually not active in many microglia cells (up to 3%) but highly specific (91%) for neurons with low a efficacy (affecting 15-17% of neurons counted). Additionally, LyzM-promoter is active in myeloid cells and peripheral tissues, according to the founder and is heavily used in the field of immunology. Cx3Cr1 promoter is highly specific to microglia targeting a large percentage of this cell type. However, we found that male Cx3Cr1-CreERT2-Dagla KO mice and control mice developed a severe hernia upon tamoxifen injections (X. Ma et al. 2015) and they could unfortunately not be used for behavioral testing. As an important consideration, it turned out, that Cx3Cr1-CreERT2-Dagla KO are not inducible by tamoxifen since uninjected mice also showed relatively high tdTomato expression in microglial cells. This, however, needs to be further validated by looking directly at the possible deletion of *Dagla* without tamoxifen; the genomic locus where RosaTomato is situated particularly accessible which might not be the case for the *Dagla* locus. The problematic of DAGLa KO in Cx3Cr1-CreERT2-Dagla KO mice not being inducible is that the KO already occurs during development (E10.5) and could therefore also affect myeloid cells in the periphery. All the above-mentioned findings concerning the mouse lines are relevant for other studies using LyzM-Cre or Cx3Cr1-CreERT2 mice to address gene functions in microglia.

Nevertheless, we did find some interesting phenotypes in LyzM-Dagla and Cx3Cr1-CreERT2-Dagla KO mice, which were surprisingly quite similar, even though the KO was affecting different cells. First, we measured body weight and food intake of the mice. While male and female LyzM-Dagla KO mice did not show any changes, female Cx3Cr1-CreERT2-Dagla KO mice showed a slight increase in food intake one day after the sucrose preference test. These data suggests, that microglial DAGLa or DAGLa on myeloid cells is involved in the metabolic regulation after palatable food intake. CB1 is also expressed in peripheral tissue, including adipose tissue, endocrine pancreas, muscle, and liver, which are all involved in energy metabolism (Bermudez-Silva, Cardinal, and Cota 2012; Cota et al. 2006; Silvestri and Di Marzo 2013). It was shown that increased CB1 signaling can increase energy storage, for instance via stimulation of fat mass expansion or glucose uptake (Vettor and Pagano 2009). Decreased circulating 2-AG, due to the deletion of *Dagla* on myeloid cells, could, therefore, decrease the efficiency of energy storage during the sucrose

preference test. Afterwards, Cx3Cr1-CreERT2-Dagla KO mice might need to eat more compared to their littermate controls to store the same amount of energy. Alternatively, there might be an effect of microglial DAGLa on processing of reward. However, since LyzM-Dagla KO mice did not show a similar phenotype, the change in food intake can rather be assigned to loss of DAGLa on microglia. Recently, microglia have indeed gained attention in the regulation of food intake and metabolism (García-Cáceres et al. 2019) The microglia in the mediobasal hypothalamus act as critical metabolic regulators (Valdearcos, Myers, and Koliwad 2019). Hypothalamic inflammation and microglial activation were discovered to be critical for a normal adaptive response to highly caloric foods (Thaler et al. 2012), moreover, this process is dependent on adequate neurogenesis in the base of the hypothalamus (McNay et al. 2012). The contribution of the endocannabinoid system in these processes is currently unknown. As both microglia and endocannabinoids regulate inflammation in the brain and cannabinoids regulate feeding and neurogenesis, this could be a promising line of study.

The body weight of female Cx3Cr1-CreERT2-Dagla KO mice was unchanged compared to control littermates. Cx3Cr1-CreERT2-Dagla KO mice did not show any changes in 2-AG, SAG or AA levels in the brain, suggesting that microglia cells are not involved in the synthesis of bulk 2-AG under normal conditions *in vivo*. However, cell culture studies have shown that microglia are producing very high amounts of 2-AG, indicating an important role of microglial 2-AG production under inflammatory conditions (microglia often turn reactive in culture) (Walter et al. 2003). To our knowledge, 2-AG production by microglial cells *in vivo* has not been studied yet, but according to our novel results, it has a less important role under normal conditions in the brain. LyzM-Dagla KO mice also did not show any changes in bulk 2-AG and related arachidonate lipidome in the brain, suggesting that the number of neurons affected by the KO in LyzM-Dagla KO mice is too low to induce changes in arachidonate remodeling. However, a previous study using the same LyzM-Dagla KO mouse line, measured endocannabinoids in the plasma of the mice and found decreased 2-AG levels in the blood ($p=0.052$), confirming the *Dagla* deletion in myeloid cells in this mouse line (Jehle et al. 2016). Apparently, myeloid cells do produce measurable amounts of 2-AG in the periphery. Nevertheless, plasma 2-AG seems not to affect the bulk 2-AG levels in the brain. Peripheral changes in 2-AG levels could still have an impact on the behavior. The data gathered for LyzM-Dagla KO mice are generally hard to interpret because of the different cell types that are affected by the KO (myeloid cells, peripheral tissues, a very small amount of microglia in the brain, and few neurons). Thus, all effects measured in this mouse line need further intensive study, which was beyond the scope of this thesis. Nevertheless, having Cx3Cr1-CreERT2-Dagla KO mice as a comparison, where only microglia cells and perhaps myeloid cells were affected by the KO, enables some conclusions. Due to these differences between the KO penetrance in the mouse lines we expected different behavioral results. In the open-field test, both lines showed normal behavior that was similar to their littermate controls. In zero-maze, female Cx3Cr1-CreERT2-Dagla KO mice spent tendentially less time ($p=0.07$) in the open compartment of the maze, they were looking down from the maze

more often and showed a stretched posture when leaving the closed compartment more often than littermate control- all behavioral changes in zero-maze are typical signs of increased anxiety (Shepherd et al. 1994). However, the phenotype is rather mild and only significant for those few parameters of the entire test. Even though the KO differs in Cx3Cr1-CreERT2-Dagla KO and LyzM-Dagla KO mice, female, but not male LyzM-Dagla KO mice showed a similar phenotype of increased anxiety in the zero-maze, also looking down less often and displaying more of the stretched posture. Since both mouse lines show similar phenotypes, this behavior can probably be assigned to the loss of DAGLa in myeloid cells. It is unlikely that DAGLa KO on microglia has an impact on anxiety since the amount of microglia affected by the KO in LyzM-Dagla KO mice is very small (around 3% in cortex and hippocampus). It might also be that the deletion of *Dagla* in microglia is stronger in the amygdala which was not checked for the KO efficacy. It is also conspicuous that LyzM-Dagla KO mice and Syn-Dagla KO mice show opposite behavior in the zero-maze, even though the in LyzM-Dagla KO mice Dagla was also deleted in some neurons; here, the different efficiency of the KO in these lines likely plays a role. Interestingly, reduced circulating concentrations of 2-AG have been also observed in the posttraumatic stress disorder (PTSD) patients, (Matthew N. Hill et al. 2013b), fitting to the hypothesis that a deletion of *Dagla* in myeloid cells might be responsible for slightly increased anxiety in our mouse models. Anxiety-like behavior was only measured in two to three parameters of one anxiety test, however. Further anxiety tests should be added to confirm the finding of increased anxiety in both microglial Cre lines.

Depression-like and maternal care behavior was unaffected in LyzM- and Cx3Cr1-CreERT2-Dagla KO mice. Only in the sucrose preference test, we found that Cx3Cr1-CreERT2-Dagla KO mice showed elevated sucrose preference compared to littermate controls, which might be related to impaired energy storage or reward-related processing again, as discussed above.

Last, we investigated adult neurogenesis in our microglial/myeloid Cre lines. Both lines showed decreased survival of NPCs 21 days after the last BrdU injection, measured by the number of BrdU-positive cells in the dentate gyrus. However, differentiation was unaffected by this decrease in survival, since the same number of newly formed astrocytes and neurons was found in both KO models compared to their littermate controls. This decrease in survival of NPCs can probably be attributed to the loss of 2-AG in the periphery again since both lines showed similar phenotypes. The dentate gyrus is a neurogenic niche that is highly influenced by peripheral signals from the blood. There is a unique vascular organization in neurogenic niches supplying NCS with nutrients and providing signals, like growth factors, that impact adult neurogenesis (Shen et al. 2008; G. J. Sun et al. 2015). Moreover, the blood-brain-barrier is leakier in areas containing NSCs to allow these cells to more easily access factors from the blood (Tavazoie et al. 2008). Since 2-AG is known to be involved in the regulation of adult neurogenesis and CB1 signaling is important for survival of NPCs (Wolf et al. 2010), decreased levels in the periphery might decrease survival of newly formed NPCs in our mouse lines. To prove this hypothesis, a bone marrow irradiation and transplantation with the KO bone marrow could be used, to see if the same changes in neurogenesis would happen

in healthy mice producing myeloid cells with *Dagla* KO. Another theory is that decreased 2-AG levels in the blood might influence the cerebrovascular blood flow in the neurogenic niches. Endocannabinoid signaling is known to induce cerebrovascular relaxation, involving inhibition of smooth muscle contractility and release of vasodilatory mediators from the endothelium (Benyó et al. 2016). In a human study, cannabis users were shown to have increased cerebral blood flow positively correlated with increased THC levels in the plasma (Mathew et al. 1992). The cerebral vasculature responds to CB1 activation with vasodilation in a dose-dependent manner (Ellis, Moore, and Willoughby 1995; Gebremedhin et al. 1999; Wagner et al. 2001) and endocannabinoids can activate CB1 located on both smooth muscles and endothelial cells of cerebral vessels (Y. Chen et al. 2000; Golech et al. 2004). Numerous observations indicate that endocannabinoids participate in the control of systemic arterial blood pressure and the blood supply to different organs, including the brain (Battista, Fezza, and Maccarrone 2005; Pál Pacher et al. 2008; Stanley and O'Sullivan 2014). Thus, it might be that decreased 2-AG in plasma of LyzM- and Cx3Cr1-CreERT2-Dagla KO mice impairs cerebral blood flow and the blood supply of the neurogenic niche, leading to decreased survival of the NPCs. Preliminary data, not shown in this thesis, already indicate a possible change of the cerebrovascular system in the dentate gyrus of Cx3Cr1-CreERT2-Dagla KO mice. Further experiments need to be performed to prove the hypothesis.

A third factor that could affect adult neurogenesis could be systemic inflammation, since inflammation is known to impact adult neurogenesis (Kohman and Rhodes 2013). 2-AG has anti-inflammatory potential and is downregulated in the plasma of LyzM-Dagla KO mice. Thus, we hypothesized that there might be a peripheral inflammation affecting neurogenesis. Several studies have found significant, positive correlations between circulating 2-AG concentrations and concentrations of the pro-inflammatory cytokine interleukin-6 (IL-6) (Knight et al. 2015; Weis et al. 2010). Therefore, the expression of inflammatory markers in LyzM-Dagla KO mice was estimated. Contrary to our hypothesis, LyzM-Dagla KO mice showed a downregulation of the expression of most pro-inflammatory cytokines, pointing rather towards a hypo-inflammation in this mouse line. Hypo-inflammation occurs for instance in sepsis, right after hyper-inflammation. It is a process that helps in restoring immune homeostasis but may lead to drastic immune suppression and higher sensitivity to secondary infections that can even lead to death (Tsirigotis et al. 2016). For our LyzM-Dagla KO mice, living in a germ-controlled barrier, this might not be a problem, but in nature, those mice would probably not survive. To our knowledge, it was not shown yet whether hypo-inflammation can also impact neurogenesis. Further investigations need to be done to study this.

A forth hypothesis is that a heterozygous deletion of the fractalkine receptor (Cx3Cr1) resulted in a reduction of hippocampal neurogenesis with reduced cognitive ability as a consequence (Bachstetter et al. 2011; Rogers et al. 2011). Furthermore, administering exogenous CX3CL1 to aging animals led to an improvement of hippocampal neurogenesis (Bachstetter et al. 2011). Since Cx3Cr1-CreERT2-Dagla KO mice carry a Cx3Cr1-CreERT2 insertion, a heterozygous deletion of

Cx3Cr1 might also affect the changes in adult neurogenesis. If the mouse line turns out to be inducible, we could use the uninjected cre-positive mouse group as control group to investigate this possibility. Since one allele of the Cx3Cr1 gene is still functional and LyzM-Dagla KO mice show a similar phenotype in adult neurogenesis this possibility is not very likely.

To conclude, the data gathered from LyzM-Dagla KO and Cx3Cr1-CreERT2-Dagla KO mice are difficult to interpret due to the pitfalls of the mouse lines mentioned above. Nevertheless, it seems like peripheral DAGLa, rather than DAGLa on microglia, has a profound effect on the survival of NPCs during adult hippocampal neurogenesis. This impaired survival is either connected to an impaired supply of endocannabinoids and other factors to the neurogenic niche through the blood or a hypo-inflammatory state that was observed in LyzM-Dagla KO mice. The bulk levels of endocannabinoids in the brain were not changed in Cx3Cr1-CreERT2-Dagla KO mice, suggesting a modest contribution of microglia to bulk brain endocannabinoid levels under normal conditions. A slight anxiety-related phenotype of both lines indicated that peripheral DAGLa might be involved in the regulation of anxiety.

4.4 Sex differences in endocannabinoid system

Sex differences in the expression of proteins of the endocannabinoid system or differences in the production of endocannabinoids might be important to explain differential effects in our behavioral experiments between males and females. Furthermore, the expression of proteins of the endocannabinoid system in males vs. females has not been extensively studied, yet. Whereas for example different effects of exogenous cannabinoids have been observed in male and female rodents and even in men and women. According to literature, women and female rodents tend to be more sensitive to the effects of exogenous cannabinoids (Cha et al. 2007; Craft et al. 2012; Mathew, Wilson, and Davis 2003).

To investigate possible sex differences in the endocannabinoid system we performed an expression analysis of proteins involved in the system in the hippocampus and cortex of male and female Dagla fl/fl control mice. Furthermore, we measured lipids in different brain regions of these mice. Female mice were controlled for their estrous cycle state and were only used for experiments in di- and metestrous phase, like was also done for the lipidomics in all other mouse lines. It was previously shown that endocannabinoid levels are changing especially in the proestrus of female mice (Bradshaw et al. 2006). In the endocannabinoid measurements, we did not find changes in 2-AG levels between male and female Dagla fl/fl mice, suggesting that 2-AG levels are similar in male and female mice when female mice are not in their behavioral estrus and ovulation phase. Furthermore, it shows that our estimation of the cycle in female mice was reliable, which is also

important for the lipidomics performed in females of all other lines. Also, the levels of DAGL substrate SAG and 2-AG metabolite AA did not differ between male and female mice. In the PFC, we measured higher anandamide levels in female Dagla fl/fl mice, a result that has not been described in literature before. While the expression of all proteins tested in the hippocampus was similar between males and females, measurements showed a sexual dimorphism in some proteins in the cortex. Both CB1 and MAGL were lower expressed in female mice compared to male mice. Lower expression of MAGL did not influence bulk 2-AG or AA levels in female mice, suggesting that the changes in MAGL mRNA transcripts are not big enough to change bulk levels at baseline or that MAGL enzyme has different activities in cortex of males and females. Lower expression of CB1 in the cortex of female mice compared to males might contribute to different effects of cannabinoids in male and female mice, especially functions depending heavily on the cortex, like motility. Additionally, higher AEA levels found in female cortex might compensate for the decreased receptor expression in the cortex of females.

In summary, female mice seem to express a lower amount of the endocannabinoid system components in the cortex, but not in the hippocampus, compared to male mice. These changes had no impact on the bulk 2-AG, precursors or metabolite levels, however, might become important when the system is challenged (e.g. a strong stressor). Lower expression of CB1 in the cortex of female mice might lead to sexual dimorphism in endocannabinoid signaling regarding cortical functions.

4.5 Conclusion and Outlook

In the last few years, the enzymes involved in the biosynthesis and degradation of endocannabinoids have emerged as important targets for the treatment of psychiatric and neurological disorders (Cristino, Bisogno, and Di Marzo 2019). In this study, we have combined the usage of conditional Dagla KO mouse lines, behavioral analysis, the mRNA detection method RNAscope, and LC-MS/MS to assess the role of cell-specific DAGLa in 2-AG production and associated arachidonate remodeling in the context of neuropsychiatric disorders and adult hippocampal neurogenesis.

Besides this, the thesis points out several considerations when using available Cre recombinase lines that might be helpful for interpreting other studies using these lines to address cell-specific deletion of proteins. Especially the LyzM-Cre line, that was used as the state-of-art model to investigate microglia-specific gene expression, turned out to be highly unspecific for microglial cells in the brain. Due to GFAP expression in NPCs, many neurons in addition to astrocytes were affected by *Dagla* deletion in GFAP-Dagla KO mice. Since several different Cre lines were used in the current

study to address one cell type, valuable conclusions could still be drawn by comparing these lines with each other.

The present study gives further support for the observations that disrupted endocannabinoid signaling is involved in the development and manifestation of anxiety disorders and depression. Moreover, it is the first to describe which cell types contribute to the endocannabinoid production relevant for these behaviors. We found that 2-AG synthesis, especially by neurons, is important to control stress levels in mice, probably by regulating negative feedback mechanisms terminating stress. Since increased stress is one of the major triggers in the development of affective disorders, this novel finding could be helpful in the understanding of the pathology of certain disorders. Additionally, the analysis of Syn-Dagla KO mice and GFAP-Dagla KO mice in this study showed that the synthesis of endocannabinoids by neurons is involved in the regulation of anxiety. Depression-like behavior, on the other hand, and concomitant changes in adult neurogenesis are according to our study mainly regulated by endocannabinoid production by astroglial cells. Importantly, the current study was the first one showing that DAGLa is actually expressed in astrocytes in the adult mouse brain *in vivo* and that astrocytic DAGLa is relevant for behavioral regulation.

This thesis also shows a drastic impact of astroglial *Dagla* deletion on maternal behavior, suggesting a possible involvement of 2-AG specifically produced by astrocytes in postpartum depression and identifies GFAP-Dagla KO and GLAST-CreERT2-Dagla KO mice as useful mouse models to study this affective disorder. Such mouse models are at the moment not available to our knowledge.

2-AG produced by astrocytes or NPCs is important for adequate adult neurogenesis. Impairments in adult neurogenesis, a known hallmark of depression, were also found in our astrocytic Dagla KO mice. Our study reveals that 2-AG produced by astroglial cells is important to promote proliferation of NPCs in the dentate gyrus. This finding might be helpful for the understanding of how impaired neurogenesis is involved in depression and the action of antidepressants, which fittingly lead to an increase in endocannabinoid levels and adult neurogenesis. More specifically, decreased DAGLa expression was accompanied with decreased expression of fibroblast-growth factor 2, a growth factor produced by astrocytes that is known to promote adult neurogenesis. It is possible that DAGLa activity might regulate the expression of this growth factor, a novel finding that needs further investigations. The question of whether adult neurogenesis is a consequence of depression or the other way around could be answered using a Cre line that specifically deletes *Dagla* in NPCs without affecting astrocytes or other cell types. However, such a Cre line is not available to date in our knowledge.

While endocannabinoid production in microglia plays a critical role in inflammatory processes, it seems to be less important under normal conditions in the mouse brain. This study gives a novel indication, that DAGLa in myeloid cells could play a role in survival of NPCs during adult hippocampal neurogenesis. It might be that decreased 2-AG production in myeloid cells leads to impairments in the cerebrovascular system. This hypothesis also needs further investigation, which

was beyond the scope of this study.

Depression and anxiety disorders are the most common affective disorders in the world and only a few drugs are available for the treatment of these disorders. Furthermore, it is not clear yet, how antidepressants elicit their therapeutic potential. Increasing endocannabinoid levels might be a promising tool to treat these psychiatric disorders. Possibilities for therapeutically targeting different cell types are at the moment being actively developed and might in the future lead to an approach for specifically affecting endocannabinoid production in certain cell types. The content of this thesis is important for the basic understanding of the endocannabinoid production in the brain and the involvement of different cell types in affective disorders.

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