Aus dem Institut für Veterinär-Physiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

und

dem Forschungsinstitut für Nutztierbiologie (FBN)
Dummerstorf

# Investigations into the endocannabinoid system and endocannabinoid-mediated control of energy homeostasis in late-lactating dairy cows

**Inaugural-Dissertation**
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
**Isabel van Ackern**
Tierärztin aus Münster

Berlin 2023
Journal-Nr.: 4395

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# List of abbreviations

| | |
|---|---|
| 2-AG | 2-Arachidonoylglycerol |
| ACC1 | Acetyl-CoA-carboxylase 1 |
| ACTH | Adrenocorticotropic hormone |
| AEA | Anandamide |
| AgRP | Agouti-related peptide |
| ANS | Autonomic nervous system |
| ARC | Arcuate nucleus |
| BBB | Blood-brain barrier |
| cAMP | Cyclic adenosine monophosphate |
| CART | Cocaine- and amphetamine-regulated transcript |
| $CB_1$ | Cannabinoid receptor 1 |
| $CB_2$ | Cannabinoid receptor 2 |
| CCK | Cholecystokinin |
| CNS | Central nervous system |
| COX | Carbohydrate oxidation |
| CRH | Corticotropin-releasing hormone |
| DAGL | Diacylglycerol lipase |
| DGAT-2 | Diacylglycerol acyl transferase-2 |
| DMN | Dorsomedial nucleus |
| ECS | Endocannabinoid system |
| ENS | Enteric nervous system |
| FAAH | Fatty acid amide hydrolase |
| FAS | Fatty acid synthase |
| FOX | Fat oxidation |
| GIT | Gastrointestinal tract |
| GLP-1 | Glucagon-like peptide 1 |
| GLP-2 | Glucagon-like peptide 2 |
| HPA axis | Hypothalamic-pituitary-adrenal axis |
| LPL | Lipoprotein lipase |
| MAGL | Monoacylglycerol lipase |
| MCH | Melanin-concentrating hormone |
| mPFC | Medial prefrontal cortex |
| NAAA | N-Acylethanolamine-hydrolysing acid amidase |
| NAPE | N-Arachidonoyl phosphatidylethanolamine |
| NAPE-PLD | NAPE-specific phospholipase D |

| | |
|---|---|
| NEFA | Non-esterified fatty acid |
| NPY | Neuropeptide Y |
| OX-A | Orexin A |
| OX-B | Orexin B |
| PCR | Polymerase chain reaction |
| POMC | Pro-opiomelanocortin |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PVN | Paraventricular nucleus |
| PYY | Peptide YY |
| SCD-1 | Stearoyl-CoA desaturase-1 |
| SREBP1c | Steroid regulatory binding protein 1c |
| THC | Δ9-Tetrahydrocannabinol |
| TRH | Thyrotropin-releasing hormone |
| VMN | Ventromedial nucleus |
| WAT | White adipose tissue |

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# Chapter 1: Introduction

Both in conventional and organic dairy farming, cows face many challenges, including production diseases such as udder inflammation (mastitis), fertility disorders, and hoof and limb diseases, which are associated with high use of antibiotics, early animal losses, and financial losses (Mulligan and Doherty 2008). Underlying factors affecting animal health and performance are husbandry and management conditions with inadequate production standards, e.g., inconsistencies in diet formulation, an unsuitable environment, an inappropriate breeding policy or various combinations of these factors (Drackley 2006; Mulligan and Doherty 2008). The period when cows are most susceptible for production disorders is the transition period from the non-lactating to the lactating state under the stress of parturition, 3 weeks before to 3 weeks after parturition (Drackley 1999; Drackley et al. 2005). During the transition period, dairy cows are exposed to enormous metabolic and endocrine changes and are often exposed to environmental stressors associated with the management of group and housing changes (Mulligan and Doherty 2008). According to Grummer (1995) prepartum feed intake decreases 30 to 35 % and exposes cows to a negative energy balance leading to a compromised lactation, health and reproduction. Therefore, disease prevention and minimizing the drop in feed intake are critically important to health, production, and profitability of dairy cows (Leblanc et al. 2006). Strategies to optimize the balance between feed intake and mobilization of body fat exist and can help to improve animal welfare (Drackley et al. 2005). However, cows often fail to adapt to these metabolic and management changes, and a better understanding of the control of feed intake, energy metabolism, and stress adaptation is needed to help prevent transition problems and lower the economic burden.

In the past years, intensive research (mostly in non-ruminants) engaged in studying a promising and multifaceted system within the body of vertebrates, the so-called endocannabinoid system (**ECS**). The ECS is involved in the regulation of a variety of metabolic processes (Ruiz De Azua and Lutz 2019), including the control of feed intake and appetite, as well as energy homeostasis (Di Marzo and Matias 2005; Osei-Hyiaman et al. 2006). As lipid-based signaling molecules, endocannabinoids occur ubiquitously in the body and exert their effects at numerous sites of action. In non-lactating non-ruminants, both peripheral and central pharmacological activation of the ECS has been shown to lead to a robust increase in feed intake (Jamshidi and Taylor 2001; Gomez et al. 2002; Kirkham et al. 2002) and support anabolic metabolism (Kunos et al. 2008). Furthermore, the ECS plays an important role in the habituation to stress and the return to a non-stressed state (Morena et al. 2016). In order to utilize these properties also in dairy cows and potentially facilitate the adaptation to metabolic challenges, a better understanding of endocannabinoid signaling is needed. Targeted activation of the ECS in dairy cows could provide the opportunity to improve feed intake and

energy metabolism during the transition period and help to manage stress after environmental changes. However, ECS research in dairy cows is still in its infancy and studies are scarce. Particularly, the direct effect of activation of the ECS has not yet been investigated in dairy cows. In order to establish an initial baseline and to exclude the influence of metabolic and endocrine changes of the transition period, only late-lactating cows were included in the present study. More specifically, the focus of the current thesis was to explore the fundamental characteristics of the ECS and its involvement in feed intake regulation and energy homeostasis in late-lactating dairy cows and to provide a basis for future studies to one day utilize the manifold possibilities of the ECS in transition cows. To this end, a large trial with several subsets was performed in late-lactating dairy cows treated with endocannabinoids, capturing numerous data as well as tissue and plasma samples for a wide range of analysis.

# Chapter 2: Literature review

## 2.1 The endocannabinoid system

The endocannabinoid system (**ECS**) is a complex network ubiquitously expressed throughout the body of all vertebrates with an essential regulatory role in numerous physiological processes (Ruiz De Azua and Lutz 2019). Interest for the ECS arose with the identification of Δ9-tetrahydrocannabinol (**THC**), one of the major active components of *Cannabis sativa* (Gaoni and Mechoulam 1964), and its role as ligand binding to cannabinoid receptors (Matsuda et al. 1990; Munro et al. 1993). Knowledge gained over time led to a fundamental understanding of the ECS, consisting of cannabinoid receptors, their endogenous ligands, and corresponding synthetic and degradative enzymes (Kilaru and Chapman 2020).

### 2.1.1 Cannabinoid receptors

So far, two specific cannabinoid receptors have been cloned and characterized, the type 1 (**$CB_1$**) and type 2 (**$CB_2$**) receptor (Matsuda et al. 1990; Munro et al. 1993), both of which are found in neural and non-neural cells. In more detail, $CB_1$ receptors are expressed at high levels in the central nervous system (**CNS**) (Herkenham et al. 1990) and are also present in the majority of peripheral organs, e.g., the adipose tissue, liver, gastro-intestinal tract, muscle, and reproductive organs (Ruiz De Azua and Lutz 2019). In contrast, central $CB_2$ receptors are expressed to a much lesser extent than $CB_1$ receptors (Gong et al. 2006) and the peripheral expression is mostly limited to immune cells and lymphoid tissues (Buckley et al. 2000).

The $CB_1$ and $CB_2$ receptors belong to the group of rhodopsin-like (class A) G protein-coupled receptors and share a 44% amino acid sequence identity (Munro et al. 1993; Montero et al. 2005). Consistent with other G protein-coupled receptors, the cannabinoid receptors consist of seven transmembrane domains with extracellular and intracellular loops, an extracellular N terminus and an intracellular C terminus (Bramblett et al. 1995). Activation leads to a cell-specific signaling cascade. Specifically, for signal transduction, the cannabinoid receptors are primarily coupled with a $G\alpha_{i/o}$ subunit. Receptor activation therefore inhibits the adenylyl cyclase activity, thus attenuating the production of cyclic adenosine monophosphate (**cAMP**) resulting in a decreased activity of the cAMP-dependent protein kinase (Howlett 2002). In addition, signal transduction through activation of mitogen-activated protein kinase, inhibition of voltage-gated calcium channels, and activation of potassium channels has been described (Pertwee et al. 2010).

Furthermore, evidence suggests the existence of additional types or subtypes of cannabinoid receptors, as some effects occur independently of $CB_1$ and $CB_2$ receptor activity

(Di Marzo et al. 2000). Regardless of a low sequence similarity to the $CB_1$ and $CB_2$ receptor, the deorphanized G protein-coupled receptor GPR55 has been shown to interact with endocannabinoids (Ryberg et al. 2007; Yin et al. 2009). However, conflicting results have been found in different cells and the pharmacology remains to be clarified (De Petrocellis and Di Marzo 2010). Other receptors under investigation are the G protein-coupled receptor GPR119 and GPR18 (Brown 2007; Console-Bram et al. 2014) and the transient receptor potential channels superfamily of cation channels (Pertwee et al. 2010).

Specific ligands for the cannabinoid receptors can be categorized as endocannabinoids and exocannabinoids, which include synthetic cannabinoids and phytocannabinoids. Focusing on the ECS, this thesis will address endogenous cannabinoid receptor ligands and synthetic agonists and antagonist used in the current research (see below).

### 2.1.2 Endocannabinoids

Endocannabinoids are naturally occurring cannabinoids produced within the body. They are synthesized from membrane phospholipids and function as bioactive lipid messenger molecules. The first discovered endogenous cannabinoid receptor ligands were N-arachidonoylethanolamide (anandamide, **AEA**) and 2-arachidonoylglycerol (**2-AG**) (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995), followed by N-arachidonoyldopamine (NADA), 2-arachidonoyl (Noladin) and O-arachidonoylethanolamine (Virhodamine) (Bisogno et al. 2000; Hanus et al. 2001; Porter et al. 2002). To date, AEA and 2-AG are the best characterized compounds and will be the main focus of this thesis.

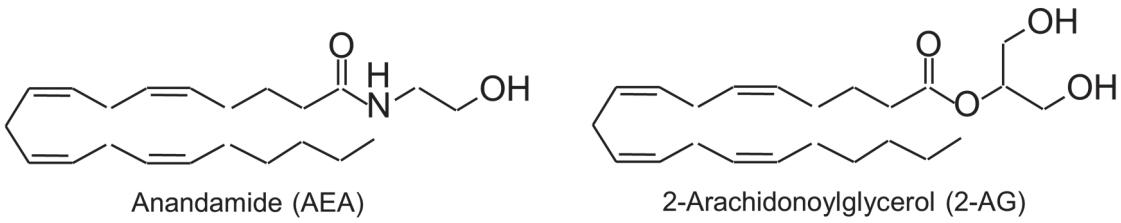


**Figure 1 |** Chemical structures of the two best studied endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (adapted from Di Marzo et al. (2004))

Among endocannabinoids, AEA functions as a partial agonist with high affinity, low efficacy for $CB_1$ and even lower efficacy at $CB_2$ receptors. On the other hand, 2-AG has lower affinity, but is a fully effective agonist at both $CB_1$ and $CB_2$ receptors (Reggio 2010).

As derivatives of arachidonic acid and highly lipophilic compounds, endocannabinoids are mainly produced “on demand” from membrane-bound precursors ubiquitously occurring within the body following physiological and pathological stimuli in the form of strong calcium influx through voltage-gated calcium channels (Piomelli 2003). In case of AEA, the major synthetic pathway derives from N-arachidonoyl phosphatidylethanolamine (**NAPE**), which is synthesized by acylation of the membrane phospholipid phosphatidylethanolamine by a calcium-dependent N-acyltransferase. In a further step, NAPE is hydrolyzed to AEA involving an NAPE-specific phospholipase D (**NAPE-PLD**, Figure 2) (Piomelli 2003; Di Marzo 2011). Enzymatic steps involved in the formation of 2-AG include the hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol by phospholipase C and the conversion of the latter to 2-AG by the membrane-bound diacylglycerol lipase (**DAGL**, Figure 2) (Piomelli 2003; De Petrocellis et al. 2004). The identity of transport mechanisms, e.g., transmembrane carriers remains controversial. However, the existence of specific transporters is speculated (Ligresti et al. 2004) and investigations into mechanisms exceeding passive diffusion are ongoing (Maccarrone et al. 2010; Piomelli and Tagne 2021). Following biosynthesis, endocannabinoids are instantaneously released in the extracellular milieu and can directly act as autocrine or paracrine ligands of cannabinoid receptors or be distributed in the bloodstream bound to serum albumin as endocrine lipid messengers (Bojesen and Hansen 2003). Evidence suggests the intracellular accumulation of AEA in adiposomes as a possible storage mechanism (Oddi et al. 2008), which could imply an extended lifespan for the otherwise very unstable molecules (Maccarrone et al. 2010). After reuptake into the cell by possible carrier-mediate facilitated diffusion (Hillard et al. 1997), AEA is primarily degraded by fatty acid amide hydrolase (**FAAH**, Figure 2) (Hillard et al. 1995) to arachidonic acid and ethanolamine. Additional enzymes regulating the biotransformation of AEA are N-acylethanolamine-hydrolysing acid amidase (**NAAA**) or cyclooxygenase-2 (Basavarajappa 2007). The majority of 2-AG is hydrolyzed by monoacylglycerol lipase (**MAGL**, Figure 2) to arachidonic acid and glycerol (Dinh et al. 2002) and to a lesser extent also by the *α*/*β*-hydrolase domain containing enzyme (ABHD) 6 and 12 (Blankman et al. 2007).

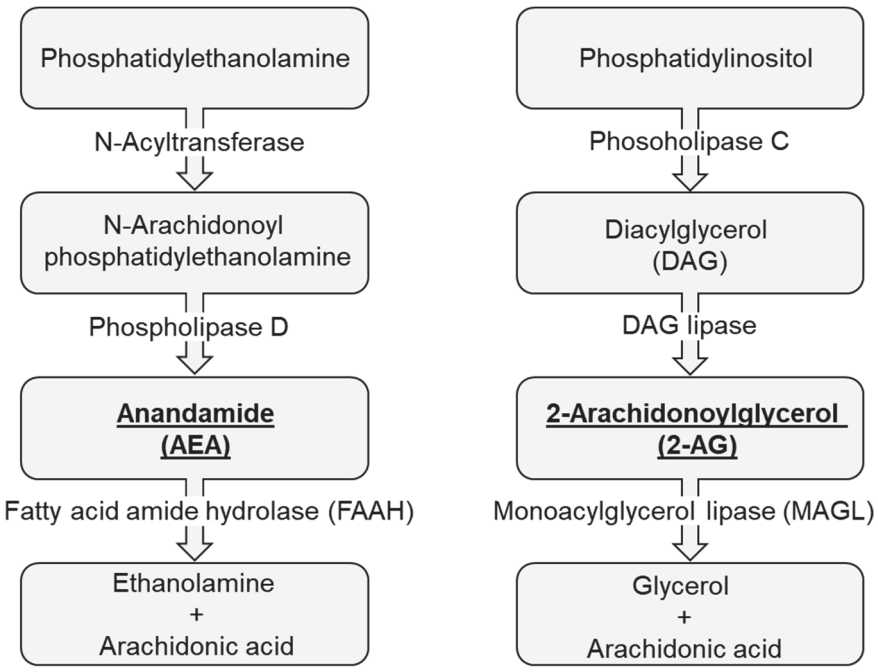


**Figure 2 |** Simplified representation of main pathways of synthesis and degradation of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (modified from El Manira and Kyriakatos (2010))

### 2.1.3 Endocannabinoid tone

The endocannabinoid tone is species- and tissue-specific and can be modulated by a number of physiological and non-physiological factors. Physiological influencing factors include, among others, the metabolic state of the body. On-demand production enables the ECS to react quickly to changes in energy supply. Accordingly, fasting increases the tissue AEA and 2-AG concentration such as in the limbic forebrain, hypothalamus, and the small intestine of rats (Gomez et al. 2002; Kirkham et al. 2002; Dipatrizio et al. 2015), whereas refeeding has been shown to immediately reverse this effect. Interestingly, the endocannabinoid tone is also generally upregulated in different stages of obesity in humans and rodents (Engeli 2008; Izzo et al. 2009), indicating a functional dysregulation and emphasizing the complexity of the underlying mechanisms. Furthermore, a stress-induced modulation of the endocannabinoid tone has also been described, leading to a decrease in AEA but an increase in 2-AG levels in the brain of rats (Hill et al. 2010b; Yasmin et al. 2020).

Endocannabinoid levels are also thought to be affected by various dietary constituents; for instance, by changing the n6/n3 ratio of the diet. The n6 fatty acid linoleic acid serves as a precursor of arachidonic acid, which in turn is a precursor to AEA and 2-AG. Therefore, feeding diets supplemented with linoleic acid (high n6/n3 ratio) can increase the endocannabinoid tone.

More specifically, elevated dietary linoleic acid contents have been shown to increase AEA and 2-AG levels in the brain, bowel, and liver tissue of mice (Alvheim et al. 2012; Alvheim et al. 2014; Ghosh et al. 2019). On the contrary, diets with higher content in n-3 polyunsaturated fatty acids have been shown to reduce the endocannabinoid tone in mice (Watanabe et al. 2003).

Pharmacologically, the endocannabinoid tone and receptor activation can be selectively up- and down-regulated. Pharmacological agents can be categorized as: (*i*) modulators of endocannabinoid formation, (*ii*) inhibitors of endocannabinoid cellular uptake, (*iii*) inhibitors of endocannabinoid degradation, and (*iv*) modulators of cannabinoid receptors (Paredes-Ruiz et al. 2021; Piomelli and Tagne 2021). Specifically, FAAH and MAGL inhibitors are commonly used in research and are proposed as possible future therapeutic targets (Paredes-Ruiz et al. 2021). Also, several selective agonists and antagonists are described and frequently used as modulators of cannabinoid receptor activity, e.g., WIN-55,212, HU-210, and CP 55,940 as agonists, or SR141716A (rimonabant) and AM-251 as antagonists to both the $CB_1$ and $CB_2$ receptor (Pertwee et al. 2010).

## 2.2 The endocannabinoid system as a modulator of energy homeostasis

Energy homeostasis, i.e., the control over the equilibrium and constancy of available energy within the body, is the key goal of numerous physiological processes and is often simplified as the balance between feed intake and energy expenditure. The ECS plays an essential regulatory role within the homeostatic control of energy balance and is involved in a vast array of physiological processes. The metabolic regulation extends from feed intake and appetite regulation on central and peripheral levels, to the integration within the neural response to stress, and to lipid and glucose metabolism (Figure 3). Over all, the ECS favors anabolic processes and facilitates feed intake, energy storage, and decreases energy expenditure. However, so far, the ubiquitous involvement of the ECS throughout the body is far from fully understood and many mechanisms are still emerging. The following chapters will review the current knowledge of the ECS in the metabolic regulation of energy homeostasis relevant to the scope of this thesis.

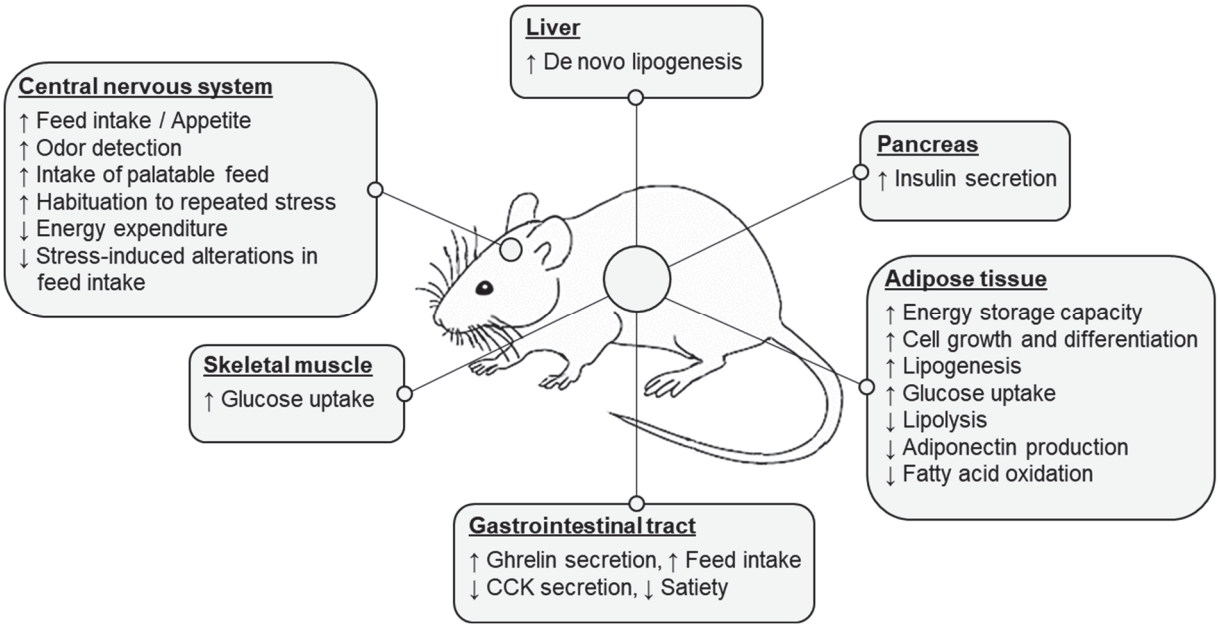


**Figure 3 |** Endocannabinoid-mediated control of energy homeostasis. Simplified representation of the main responses after activation of endocannabinoid signaling in central and peripheral tissues (modified from Bellocchio et al. (2008a) and Ruiz De Azua and Lutz (2019))

### 2.2.1 Regulation of feed intake

Feed intake is controlled by a complex and precise combination of peripheral and central regulatory stimuli and can be differentiated into homeostatic feeding, the need to eat to meet caloric and nutritional requirements, and hedonic feeding, the desire to eat (Saper et al. 2002). The balance between hunger and satiety is regulated by the integration of various humoral and neuronal signals in the CNS. Central structures involved in the control of energy homeostasis include the hypothalamus, mesolimbic system, and brainstem, with the hypothalamus taking a superordinate role in signal integration (Wynne et al. 2005). Specific hypothalamic nuclei coordinating homeostatic feeding are the arcuate (**ARC**) and ventromedial (**VMN**) nuclei located in the ventromedial hypothalamus, the dorsomedial (**DMN**) and paraventricular (**PVN**) nuclei, alongside the lateral hypothalamic area (Figure 4). Neurons located in the ARC detect and integrate circulating metabolic signals and produce both orexigenic and anorexigenic neuropeptides and neuromodulators. In particular, the orexigenic neuropeptide Y (**NPY**) and agouti-related peptide (**AgRP**) (Morton and Schwartz 2001), and the anorexigenic pro-opiomelanocortin (**POMC**) and cocaine- and amphetamine-regulated transcript (**CART**) (Kristensen et al. 1998). These ARC neurons project to a number of second-order nuclei such as the PVN, VMN, and lateral hypothalamus (Cone et al. 2001). The lateral hypothalamus contains two orexigenic neuronal types that express melanin-concentrating hormone (**MCH**)

and orexins (**OX-A** and **OX-B**) (Qu et al. 1996; Burdakov et al. 2003). Neurons located in the PVN express the peptide transmitters corticotropin-releasing hormone (**CRH**), thyrotropin-releasing hormone (**TRH**) and oxytocin (Wynne et al. 2005).

Beyond homeostatic needs, circuits processing reward, motivation, and sensory properties of feed also play an integral role in feeding behavior. In particular, a series of interconnecting circuits linking the prefrontal cortex, amygdala, nucleus accumbens, hippocampus, and ventral tegmental area connect them to several hypothalamic feeding circuits via projections (Kelley et al. 2005). In order to integrate peripheral signals centrally, communication between the gastrointestinal tract (**GIT**) with its enteric nervous system (**ENS**) and the CNS is essential. The nerval portion of the gut-brain axis is composed of afferent and efferent parasympathetic and sympathetic fibers (Konturek et al. 2004). Peripheral inputs to initiate or terminate feeding include intestinal hormones and gastric stretch. The humoral portion of the gut-brain axis involves circulating hormones and metabolites, e.g., glucose, amino acids, and fatty acids. The best studied gastrointestinal hormones involved in energy intake are the anorexigenic cholecystokinin (**CCK**), peptide YY (**PYY**), and glucagon-like peptide 1 (**GLP-1**) and 2 (**GLP-2**), and the orexigenic ghrelin (Wynne et al. 2005). Besides, so-called adiposity signals such as leptin, insulin and adiponectin reach the hypothalamus via the circulation and cross the blood-brain barrier (**BBB**) to lower feed intake based on levels of stored energy (Wynne et al. 2005).

Within this complex interplay of neuromodulatory systems, the ECS, especially the $CB_1$ receptor, assumes a critical influential role (Richard et al. 2009; Koch 2017; Lau et al. 2017). With increasing research, the importance of the ECS in both central and peripheral feed intake regulation emerged (Jamshidi and Taylor 2001; Gomez et al. 2002; Kirkham et al. 2002). In general, activation of the ECS increases feed intake, while inhibition of its activity reduces feed intake (Di Marzo and Matias 2005).

Centrally, endocannabinoids are key modulators of synaptic function, primarily acting as retrograde messengers suppressing transmitter release at both excitatory and inhibitory synapses (Alger 2002; Kano et al. 2009; Castillo et al. 2012). The $CB_1$ receptors are widely expressed throughout the brain including the ARC, PVN, VMN, and LH, key regions in energy balance regulation (Herkenham et al. 1990; Wittmann et al. 2007). The majority of $CB_1$ receptors are located at presynaptic terminals, where their activation influences the release of classical neurotransmitters such as GABA or glutamate (Schlicker and Kathmann 2001). However, the underlying molecular and cellular mechanisms of central $CB_1$ signaling are far from being fully understood. In any case, it is evident that intracerebral injections of AEA and 2-AG promote energy intake (Jamshidi and Taylor 2001; Kirkham et al. 2002; Chapman et al. 2012) by activating distinct hypothalamic neurons; reflected by an increase in c-Fos immunoreactivity (Soria-Gómez et al. 2007). In fact, pharmacological stimulation of $CB_1$

receptors has been shown to affect the expression of orexigenic and anorexigenic neuropeptides within the ARC, and endocannabinoids seem to be involved in the release of NPY. A study using rat hypothalamic explant models demonstrated $CB_1$ receptor stimulation with either AEA or CP 55,940 increased hypothalamic NPY release, whereas blockage with AM251 inhibited the release (Gamber et al. 2005). Morozov et al. (2017) imply an endocannabinoid-mediated retrograde disinhibition via $CB_1$-containing axons innervating AgRP/NPY neurons by demonstrating that CB1-positive synapses innervate said neurons using 3D reconstruction of serial ARC sections of mice. Furthermore, $CB_1$ receptors have been found in a large population of POMC neurons (Koch et al. 2015) and receptor activation has been shown to downregulate POMC expression and α-MSH release in the ARC of mice (Morello et al. 2016). Moreover, data from a study by Jo et al. (2005) indicate that presynaptic $CB_1$ receptor activation leads to a suppressed inhibitory tone in perifornical neurons in the lateral hypothalamus. Also, neuromodulatory effects of endocannabinoids are intertwined with the orexin system, with a possible potentiating effect in brain regions modulating feed intake. The potential interaction between the ECS and the OX system has been demonstrated in several studies. Specifically, colocalization and heterodimerization was found in various cell lines, (Hilairet et al. 2003; Ellis et al. 2006; Jäntti et al. 2014; Imperatore et al. 2016), but most importantly also in the lateral hypothalamus and ventral striatum of mice (Cristino et al. 2013; Kim et al. 2021). Furthermore, endocannabinoid-mediated $CB_1$ activation enhanced OX-A release by reducing the inhibition of respective neurons (Cristino et al. 2013), whereas intracerebroventricular injections of the inverse $CB_1$ agonist AM251 decreased the expression of OX-A in the hypothalamus of rats and reduced feed intake (Merroun et al. 2015). Additionally, the ECS has been implicated to affect the motivation to consume palatable foods (Higgs et al. 2003; Dipatrizio and Simansky 2008) and increase odor detection in the olfactory system (Soria-Gomez et al. 2014a; Soria-Gomez et al. 2014b), thereby further promoting energy intake. Also playing an important role in the control of feed intake is the cross talk and mutual regulation between the ECS and the appetite-suppressant leptin with its hypothalamic receptors (Di Marzo et al. 2001). Hypothalamic endocannabinoid concentrations seem to be inversely correlated with circulating levels of leptin since i.v. leptin administration inhibits endocannabinoid biosynthesis in the brain, while impaired leptin signaling leads to permanently elevated hypothalamic endocannabinoid levels (Di Marzo et al. 2001).

Peripheral mechanisms of endocannabinoid signaling are also not fully understood. However, intraperitoneally (i.p.) administered endocannabinoids have been repeatedly shown to induce hyperphagia and increase energy intake in rodents (Hao et al. 2000; Gomez et al. 2002; Avraham et al. 2017). It has been shown that peripherally administered endocannabinoids may enter the bloodstream, rapidly cross the BBB (Fride and Mechoulam 1993), and activate hypothalamic neurons (Wenger et al. 1997), or indirectly and directly trigger

signaling of the gut-brain axis (Dipatrizio 2021). Indirect mechanisms of endocannabinoid-mediated gut-brain signaling include interactions with gut-derived peptides, e.g., CCK and ghrelin (Dipatrizio 2021). Argueta et al. (2019) suggested that peripheral $CB_1$ receptor activation might control feed intake by blocking the nutrient-induced secretion of the satiation peptide CCK, thereby reducing vagal afferent neural activity and increasing feed intake. In fact, they were able to demonstrate that $CB_1$ receptors are expressed in CCK-containing cells in the upper small-intestinal epithelium of mice and that receptor stimulation with WIN-55,212 inhibited fat-induced CCK release (Argueta and Dipatrizio 2017; Argueta et al. 2019). Further evidence points to a $CB_1$ receptor-mediated control of ghrelin production in the stomach (Edwards and Abizaid 2016). Direct $CB_1$ receptor activation with i.p. injections of either AEA or CP 55,940 stimulated ghrelin secretion from gastric X/A-like cells and increased plasma ghrelin concentration in rats (Zbucki et al. 2008), whereas the endocannabinoid antagonist rimonabant had opposite effects on ghrelin plasma levels (Cani et al. 2004). A series of studies also suggest that endocannabinoids directly affect the gut-brain neurotransmission via $CB_1$ receptors located on vagal afferent neurons (Burdyga et al. 2004; Burdyga et al. 2010; Berland et al. 2022). Respective neurons also express receptors for CCK and OX, and hence, $CB_1$ receptor expression and distribution was further affected by the state of satiety (Burdyga et al. 2004). However, many gaps remain in the understanding of the precise neural and molecular mechanisms by which the ECS regulates feed intake and further investigation is required to fully comprehend the complexity of the ECS.

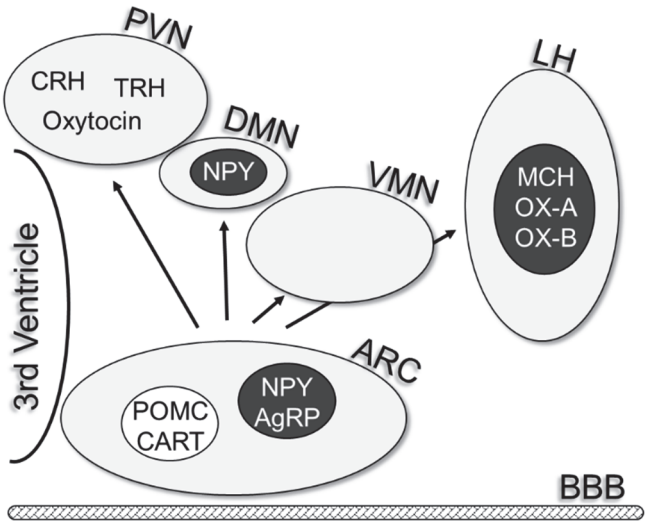


**Figure 4 |** Hypothalamic centers controlling appetite. Schematic representation of orexigenic (●) and anorexigenic (○) neurons (modified from (Wynne et al. 2005)).
Abbreviations: BBB – blood-brain barrier; ARC – arcuate nucleus; DMN – dorsomedial nucleus; VMN – nucleus of the hypothalamus; PVN – paraventricular nucleus; LH – lateral hypothalamic area; NPY – neuropeptide Y; AgRP – agouti-related peptide; POMC – pro-

opiomelanocortin; CART – cocaine- and amphetamine-regulated transcript; MCH – melanin-concentrating hormone; OX-A/B – orexin A/B; CRH – corticotropin-releasing hormone; TRH - thyrotropin-releasing hormone

### 2.2.2 Regulation of the stress response in relation to feed intake

The body's response to stress is multifaceted and effects are of profound complexity. The stress response comprises of a biological cascade of events that occur after an actual or anticipated threat to homeostasis to maintain physiologic integrity. The response to a stressor is mediated by a set of interlocking circuits in the limbic forebrain, the hypothalamus, and the brainstem (Ulrich-Lai and Herman 2009). The most immediate response includes the activation of the autonomic nervous system (**ANS**) and the release of catecholamines within the brain and circulation (Ulrich-Lai and Herman 2009). Furthermore, stress activates the neuroendocrine hypothalamic-pituitary-adrenal (**HPA**) axis resulting in the synthesis of CRH in the PVN stimulating the release of adrenocorticotropic hormone (**ACTH**) from the anterior pituitary gland which in turn triggers the synthesis of glucocorticoids in the adrenal cortex (Maniscalco and Rinaman 2017). The HPA axis functions as an important mediator in energy storage and mobilization to meet metabolic demands under baseline conditions and in response to homeostatic challenges (Maniscalco and Rinaman 2017).

The complex relationship between stress and feed intake depends on a variety of factors such as stressor type, duration, frequency, and biological and environmental context (Ulrich-Lai et al. 2015). Hence, both stress-reducing and -inducing effects on feed intake are described in literature (Ulrich-Lai et al. 2015). In animal models, stress is known to reduce feed intake in response to a wide range of stressors (Maniscalco and Rinaman 2017). However, the neural mechanism through which stress inhibits feed intake is not entirely clear. Harris (2015) described negative effects of the HPA axis on appetite and feeding behavior via activation of CRH receptors. Furthermore, Maniscalco et al. (2012) propose a link between circuits that inhibit feed intake in response to satiety signals and stress through the recruitment of phenotypically distinct, but anatomically linked, populations of hindbrain neurons.

The ECS not only regulates homeostatic and hedonic feeding (see above), but it is also integrated in the neural response to stress (Hill et al. 2010b; Hill and Tasker 2012; Morena et al. 2016; Henson et al. 2021). In general, stress exposure dynamically and temporally modulates the endocannabinoid tone by reducing AEA and increasing 2-AG concentrations in the hypothalamus and multiple limbic brain regions (see below). This bidirectional regulation occurs both after acute and chronic stress (Morena et al. 2016); however, effects appear to be amplified following chronic exposure to the same stressor (Morena et al. 2016). In rodents, the commonly used stressor, restraint stress, causes a robust reduction of AEA levels in the

amygdala (Patel et al. 2005; Rademacher et al. 2008; Hill et al. 2009), medial prefrontal cortex (**mPFC**) (Rademacher et al. 2008), and hypothalamus (Hill et al. 2010b). The decline in AEA content occurs immediately with the onset of stress (Patel et al. 2005; Rademacher et al. 2008; Hill et al. 2009). This rapid reduction is likely mediated by an increase in CRH signaling leading to an enhanced activity of FAAH, which in turn increases the AEA hydrolysis rate (Hill et al. 2009; Gray et al. 2015; Gray et al. 2016). In contrast, the elevation of the 2-AG content occurs with a time delay and increases progressively (Morena et al. 2016). Similar to AEA, restraint stress has been shown to increase 2-AG levels in the amygdala (Patel et al. 2005; Rademacher et al. 2008; Hill et al. 2010b), mPFC (Rademacher et al. 2008) and hypothalamus (Patel et al. 2004) of rodents. The delayed 2-AG elevation seems to be a subsequent result of increased corticosteroid levels (Hill et al. 2010a; Gray et al. 2016), which have been shown to downregulate the expression of the primary 2-AG-degrading enzyme MAGL (Sumislawski et al. 2011). Consistently, Roberts et al. (2012) could show a positive correlation between serum corticosterone and 2-AG concentrations in the mPFC, hippocampus, and amygdala of mice after repeated stress. However, the specific mechanisms for the divergent regulation of AEA and 2-AG still need further clarification.

In their review, Morena et al. (2016) elucidated in detail the functional role of endocannabinoid signaling in the stress response and emphasized the importance of the ECS in the return to a non-stressed state and the habituation to repeated or ongoing stress. Specifically, 2-AG appears to constrain the effects of stress and facilitate the termination of the neurobiological stress response through a negative-feedback inhibition of the HPA axis (Morena et al. 2016). In fact, pharmacological augmentation of endocannabinoid signaling has been shown to attenuate the response to restraint-induced corticosterone release in rodents (Patel et al. 2004; Hill et al. 2009; Surkin et al. 2018). In more detail, microinjections of the $CB_1$ receptor agonist HU-210 or the FAAH inhibitor URB597 into the basolateral amygdala (Hill et al. 2009) or i.p. pretreatment with either the $CB_1$ receptor agonist CP 55,940 or URB597 significantly decreased or eliminated restraint-induced corticosterone release (Patel et al. 2004). A recent study by Sticht et al. (2019) tested positive implications of systemic and central pharmacological activation of the ECS on stress-induced alterations in feed intake in rats, especially the FAAH inhibitor PF-04457845 could attenuate stress-induced hypophagia when given i.c.v. Furthermore, Bluett et al. (2017) showed that administration of the MAGL inhibitor JZL-184, accompanied by a systemic rise in 2-AG, promoted stress-resilience and increased feed consumption after stress exposure. The latter studies outline future possibilities that pharmacological activation of the ECS may counteract the stress-induced suppression of feed intake in animals.

### 2.2.3 Regulation of whole-body energy metabolism

To predict whole-body energy metabolism of an organism, gas exchange measurements in respiration chambers are a valuable technique. The $O_2$ consumption and $CO_2$ production serve as a basis for calorimetric calculations to estimate whole-body carbohydrate oxidation (**COX**), whole-body fat oxidation (**FOX**), and, when urinary nitrogen excretion is assessed, whole-body energy expenditure (Frayn 1983). So far, only few studies have investigated the overall effect of the ECS on whole-body energy metabolism with the use of respiration chambers and indirect calorimetry in rodents. Respective studies mainly draw conclusions by investigating the impact of the $CB_1$ receptor antagonist rimonabant in rats (Herling et al. 2008; Kunz et al. 2008) or the effect of $CB_1$ receptor knock out in mice (Cardinal et al. 2012; Ruiz De Azua et al. 2017). Blockage or depletion of $CB_1$ receptors resulted in an increase in whole-body energy expenditure and FOX and a reduction in COX. However, the scarcity of studies using receptor agonists leaves the effects of direct ECS activation open for interpretation, and the expected effects of decreased whole-body energy expenditure and FOX as well as an increase in COX require further investigation. In the past, more insights have been generated at the molecular level, with a focus on tissues involved in energy metabolism (see chapters below).

### 2.2.4 Regulation of lipid metabolism

Among homeostatic processes, the adipose organ is crucial for maintaining energy and metabolic homeostasis. Lipid metabolism includes the synthesis and degradation of lipids for energy generation, as well as the synthesis of structural and functional lipids, e.g., cell membranes (lipid bilayer) and bioactive compounds (hormones or hormone-like substances). Besides carbohydrates, lipids are the most important form of energy storage in the body. The body obtains energy from the breakdown of consumed dietary fats or from lipid stores during periods of restricted nutrient supply or high-energy demand. The excess of consumed energy is mainly stored as triglycerides within the white adipose tissue (**WAT**).

The role of the ECS in lipid metabolism is not completely understood, but intensive research has uncovered important mechanisms of ECS involvement in various adipose tissue functions to promote energy accumulation (Jung et al. 2021; Rakotoarivelo et al. 2021). The ECS has a profound influential role (Matias et al. 2016) particularly in WAT, which provides an important source for endocannabinoid synthesis (Buch et al. 2021). After identifying $CB_1$ receptors in rodent and human adipocytes (Cota et al. 2003; Roche et al. 2006), it became evident that cells also express functional enzymes of the ECS (Matias et al. 2006b; Spoto et al. 2006). Expression of $CB_2$ receptors has also been documented in human adipocytes

(Roche et al. 2006), but its purpose is not yet established. Among others, it has been proposed that the ECS modulates adipogenesis and has a possible stimulatory role in the differentiation from adipose progenitor cells to mature adipocytes, enhancing the capacity for storing energy. More specifically, it has been shown that $CB_1$ expression and binding efficiency progressively increases during the differentiation process (Engeli et al. 2005; Roche et al. 2006; Gasperi et al. 2007). Furthermore, receptor activation with the $CB_1$ agonist WIN-55,212 can stimulate $CB_1$ expression in mouse pre-adipocytes (Bellocchio et al. 2008b). In addition, AEA has been shown to induce adipocyte differentiation by inducing transcriptional activation of peroxisome proliferator-activated receptor gamma (**PPAR*γ***), a known marker for adipogenesis, in primary adipocyte culture and 3T3-L1 cells (Bouaboula et al. 2005; Karaliota et al. 2009). Bellocchio et al. (2008b) also demonstrated that WIN-55,212 was able to stimulate adipocyte proliferation in 3T3-L1 pre-adipocytes and increase the intracellular lipid amount, similarly to Bouaboula et al. (2005), who also showed lipid droplet accumulation in the cytoplasm of mouse pre-adipocytes. On the other hand, the $CB_1$ receptor antagonist rimonabant (SR141716) prevented these effects (Matias et al. 2006b) and had an inhibitory effect on cell proliferation and cell growth in mouse pre-adipocytes (Gary-Bobo et al. 2006; Bellocchio et al. 2008b). Moreover, the ECS is not only involved in white adipocyte proliferation and differentiation, but also plays a role in regulating lipogenesis by modulating lipoprotein lipase (**LPL**) activity, a key enzyme regulating triglyceride hydrolysis and fat storage. Cota et al. (2003) were the first to demonstrate that stimulation of $CB_1$ receptors with WIN-55,212 increased LPL activity in primary mouse adipocyte cultures from WAT, whereas pre-incubation with rimonabant blocked this effect. Similarly, Bellocchio et al. (2008b) showed an increased expression of LPL in 3T3-L1 adipocytes treated with WIN-55,212. They further showed increased expression of the enzymes fatty acid synthase (**FAS**), stearoyl-CoA desaturase-1 (**SCD-1**), and diacylglycerol acyl transferase-2 (**DGAT-2**), indicating a stimulation of fatty acid synthesis, fatty acid desaturation, and triglyceride biosynthesis. In a recent study by Buch et al. (2021) a further mechanism by which the ECS limits fat mobilization was described. Elevation of the ECS tone in WAT induced a decrease in lipolysis in both in vivo and in vitro experiments, possibly through the activation of the PI3K/Akt signaling pathway. Furthermore, the ECS modulates the synthesis and release of adipokines produced in WAT, specifically, adiponectin, which is known to enhance insulin sensitivity and increase fatty acid oxidation and glucose uptake (Yamauchi et al. 2002). Activation of $CB_1$ receptors with HU-210 or WIN-55,212 in 3T3-F442A or 3T3-L1 adipocytes, respectively, resulted in downregulation, whereas treatment with rimonabant increased adiponectin expression and release (Matias et al. 2006b; Bellocchio et al. 2008b). In line with the stimulatory effect on lipogenesis, $CB_1$ receptor activation also facilitates insulin-stimulated glucose entry in 3T3-L1 and human adipocytes (Gasperi et al.

2007; Pagano et al. 2007), a step essential for fatty acid synthesis and triglyceride biosynthesis.

The presence of $CB_1$ receptors, albeit in low density, has also been described in hepatocytes of mice (Osei-Hyiaman et al. 2005). In monogastric species, the liver represents the main source of de novo fatty acids synthesis. Osei-Hyiaman et al. (2005) reported that $CB_1$ receptor activation with HU-210 caused a more than 2-fold increase in the rate of hepatic fatty acid synthesis and an increased expression of the lipogenic transcription factor steroid regulatory element binding protein 1c (**SREBP1c**) and its targets acetyl-CoA-carboxylase 1 (**ACC1**) and FAS to promote lipogenesis and decrease fatty acid oxidation.

Overall, activation of the ECS in cells associated with lipid metabolism stimulates anabolic pathways and promotes energy conservation (Figure 3).

### 2.2.5 Regulation of glucose metabolism

This chapter is not directly within the main research focus of this thesis. However, to provide a more comprehensive overview of the involvement of the ECS in the regulation of carbohydrate metabolism, the principal role of the ECS in glucose metabolism is briefly summarized below.

Carbohydrates ingested with feed are converted into glucose, which is then distributed to tissue cells where it is broken down to provide energy. In case of a surplus of the immediate metabolic demand, glucose can be stored as glycogen in the liver and muscle and can be mobilized quickly as a short-term source of energy when needed. The major regulators of glucose metabolism are insulin and glucagon, responsible for maintaining blood glucose levels (Woods et al. 2006).

Emerging research shows that the ECS contributes to glucose utilization and insulin sensitivity, promoting energy conservation. In addition to increasing glucose uptake in adipocytes (see 2.2.4), Eckardt et al. (2009) showed that high doses of AEA increased basal and also insulin-stimulated glucose uptake in human skeletal muscle cells. The ECS was also identified in the endocrine islet cells of the pancreas (Li et al. 2011). Specifically, endocannabinoids have been shown to be involved in the regulation of cell proliferation during pancreatic islet formation; subsequently impacting glucagon and insulin secretion (Malenczyk et al. 2015). Furthermore, $CB_1$ receptor activation stimulated insulin secretion in pancreatic β-cell lines from mice (Matias et al. 2006b; Malenczyk et al. 2013). However, underlying mechanisms need further clarification and require additional research to fully understand the role of the ECS in glucose metabolism. In addition to lipids and carbohydrates, proteins also

provide energy; however, the role of the ECS in protein metabolism is uncertain and therefore not further mentioned in this thesis.

## 2.3 The endocannabinoid system in dairy cows

To date, information about the role of the ECS in dairy cows is scarce and only few studies have addressed this topic. In particular, the impact of the endocannabinoid tone in the regulation of feed intake as a possible improvement strategy for dairy health has not been sufficiently explored. So far, a few pioneer studies have describe the endocannabinoid concentration in plasma and adipose tissue of periparturient cows (Zachut et al. 2018; Kuhla et al. 2019) and have investigated ECS-related gene expression in the liver, adipose tissue, and hypothalamus (Khan et al. 2012; Zachut et al. 2018; Kuhla et al. 2019; Dirandeh et al. 2020).

The ECS plays a critical role in the physiological adaptation to major metabolic changes. In cows, the transition period from the non-lactating to the lactating state under the stress of parturition, in particular, presents a severe metabolic challenge to the overall health and productivity and is therefore of substantial research interest. Consequently, Khan et al. (2012) examined the hepatic ECS during the first two weeks postpartum when rates of hepatic fatty acid oxidation and ketogenesis are at their highest. The study was performed in relation to the dietary energy concentration since the energy content of the prepartum diet influences the level of negative energy balance postpartum and thus influences the mobilization of fatty acids from adipose tissue (Drackley et al. 2001). Findings by Khan et al. (2012) showing altered hepatic gene expression between feeding a moderate and low energy diet prepartum suggest that the ECS in the bovine liver, as in non-ruminants, might also be associated with hepatic lipid metabolism. Also focusing on the transition period, Zachut et al. (2018) were the first to describe the presence of key elements of the ECS in the subcutaneous adipose tissue of periparturient dairy cows and demonstrated that the adipose ECS is involved in the adaptation of lipid metabolism related to the onset of lactation. In particular, biopsies from adipose tissue pre- and postpartum from high and low body weight loss cows revealed two-fold elevated AEA and 2-AG levels with the onset of lactation (4 days postpartum) in the high body weight loss animals. In addition, mRNA expression of the $CB_1$ and $CB_2$ receptors tended to be greater in cows exhibiting high rates of lipolysis and inflammation postpartum. Therefore, Zachut et al. (2018) postulated increased activation of the ECS, along with higher lipolysis and signs of increased inflammation, point to a protective mechanism of the ECS to counteract this. A study by Dirandeh et al. (2020) supports this assertion by linking the degree of body condition score loss and inflammation to the activation of the ECS in the subcutaneous adipose tissue in cows

at the onset of lactation. Kuhla et al. (2019) also led to further understanding of the ECS in transition dairy cows. In two experiments, the authors explored the regulation of plasma AEA and 2-AG concentrations during transition from late pregnancy to early lactation as well as the expression of the ECS in the PVN and ARC of the hypothalamus of late- and early-lactating cows. Overall, plasma endocannabinoid concentrations increased 2.2- to 2.4-fold during early lactation but increased faster in cows exhibiting higher body fat mobilization. This again shows the interaction and possible counteractive role for endocannabinoids in relation to fat mobilization. In the hypothalamus, Kuhla et al. (2019) showed an upregulation of NAPE-PLD and $CB_1$ receptor expression, and downregulation of FAAH in the PVN of early-lactating cows compared to late-lactating cows, suggesting an increased AEA tone coinciding with a negative energy balance. In addition, a current review by Myers et al. (2021) highlights the importance of the ECS-involvement in adipose tissue metabolism and feed intake of cows, urging the need for further research on this topic. Myers et al. (2021) discusses the potential of modulating the ECS activity in periparturient dairy cows to one day improve dairy health and productivity through promoting feed intake and energy partitioning by adipocyte proliferation, lipid accumulation, and suppressing lipolysis and adipose tissue inflammation.

Other studies in ruminants have addressed the involvement of the ECS in reproductive organs. Gervasi et al. (2013) described fluctuating endocannabinoid levels in the oviduct of cows during the oestrous cycle, and endocannabinoids have been reported to act as inflammatory markers in endometrial tissue of Holstein cows (Bonsale et al. 2018). However, the role of the ECS in reproductive physiology will not be assessed in this thesis.

# Chapter 3: Aims of this thesis

In recent years, a plethora of studies has provided insights into the endocannabinoid system (ECS) in non-lactating, non-ruminant species. These studies have uncovered the comprehensive involvement of the ECS in the control of energy homeostasis, specifically its key role in regulatory processes, e.g., feed intake and appetite regulation; integration within the neural response to stress; and lipid and glucose metabolism. However, the ECS and underlying mechanisms in lactating dairy cows are still mostly unknown but could provide valuable insights to explore its therapeutic potential for metabolically imbalanced cows in the future.

The aim of this thesis was to further characterize the fundamentals of the ECS in dairy cows and to investigate its involvement in the regulatory processes of energy homeostasis by administering the two major endocannabinoids N-arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). More specifically, a series of experiments were conducted with non-pregnant Simmental cows in late lactation to investigate

1) the changes of the circulatory endocannabinoid tone in response to feeding, stress, diet composition, and i.p. endocannabinoid administrations
2) the effects of intraperitoneally (i.p.) administered endocannabinoids on feed intake, hypothalamic orexigenic signaling and stress-induced hypophagia,
3) the influence of administered endocannabinoids on whole-body and plasma lipid metabolism.

To approach the first aim a subset of experiments was conducted and dairy cows were subjected to: (*i*) short-term feed deprivation and refeeding; (*ii*) exposure to isolation stress induced by transfer from a free-ranging barn to a respiration chamber; (*iii*) different diets differing in the n6/n3 ratio; and (*iv*) i.p. injections of AEA and 2-AG, with subsequent analysis of plasma endocannabinoid concentrations.

The second aim was met by continuous recording of feed intake with and without endocannabinoid treatment and exposure to stress stimuli such as social and tactile isolation, and tethering. Hypothalamic orexigenic signaling after i.p. AEA and 2-AG administrations was analyzed in tissue samples from the paraventricular nucleus (PVN), the arcuate nucleus (ARC), and the lateral hypothalamus.

In order to address the third aim, cows were kept in respiration chambers. Gas exchange was recorded and multiple plasma samples were analyzed for plasma lipid concentrations.

# Chapter 4: A Role for Peripheral Anandamide and 2-Arachidonoylglycerol in Short-Term Food Intake and Orexigenic Hypothalamic Responses in a Species with Continuous Nutrient Delivery

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# A Role for Peripheral Anandamide and 2-Arachidonoylglycerol in Short-Term Food Intake and Orexigenic Hypothalamic Responses in a Species with Continuous Nutrient Delivery

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**Abstract:** The endocannabinoid system (ECS) plays a pivotal role in the complex control and regulation of food intake. Pharmacological ECS activation could improve health in energy-deficient stages by increasing food intake, at least in intermittent feeders. However, knowledge of the mechanism regulating appetite in species with continued nutrient delivery is incomplete. The objectives of this pilot study were to investigate the effect of the intraperitoneal (i.p.) administration of the endocannabinoids (ECs) anandamide (AEA) and 2-arachidonoylglycerol (2-AG) on food intake, plasma EC concentrations and hypothalamic orexigenic signaling, and to study how the circulatory EC tone changes in response to short-term food deprivation in dairy cows, a species with continuous nutrient delivery. The administration of EC resulted in higher food intake during the first hour after treatment. Plasma AEA concentrations were significantly increased 2.5 h after AEA injection, whereas plasma 2-AG concentrations remained unchanged 2.5 h after 2-AG injection. The hypothalamic immunoreactivity of cannabinoid receptor 1, agouti-related protein, and orexin-A was not affected by either treatment; however, neuropeptide Y and agouti-related protein mRNA abundances were downregulated in the arcuate nucleus of AEA-treated animals. Short-term food deprivation increased plasma 2-AG, while plasma AEA remained unchanged. In conclusion, i.p.-administered 2-AG and AEA increase food intake in the short term, but only AEA accumulates in the circulation. However, plasma 2-AG concentrations are more responsive to food deprivation than AEA.

**Keywords:** endocannabinoids; food intake regulation; hypothalamus; immunohistochemistry



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## 1. Introduction

The control and regulation of food intake is a complex process involving central and peripheral signals that are integrated at the brain level [1,2]. Endocannabinoids (ECs) may act at both peripheral and central sites, thus exerting a pivotal role in regulating energy homeostasis [3,4]. The endocannabinoid system (ECS) consists of the G protein-coupled cannabinoid receptors 1 ($CB_1$) and 2 ($CB_2$) [5], their endogen ligands N-arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglyerol (2-AG) [6,7] and specific enzymes for their synthesis and degradation [8]. In the brain, $CB_1$ receptors are, among others, expressed in areas controlling food intake, such as the paraventricular nucleus (PVN) of the hypothalamus [9]. In peripheral organs, $CB_1$ is predominantly expressed in the enteric nervous system of the intestine and at terminal ends of the vagus nerve [10,11], thus enabling ECs to engage in afferent communication along the gut–brain axis [12,13]. As derivatives of arachidonic acid, ECs are produced ubiquitously within the body. Bound to serum albumin [14], ECs can be distributed throughout the body via the circulation and in the case of AEA, be stored in intracellular adiposomes [15,16]. The cellular uptake and storage of AEA potentially extends the previously reported short half-life

of circulatory 2-AG and AEA of only a few minutes [17,18]. AEA and 2-AG function as lipophilic signaling molecules and modulate the release of various neurotransmitters [19]. In rodents, intraperitoneally administered ECs have been shown to induce hyperphagia and increase energy intake [20–22] by activating vagal [12] and hypothalamic $CB_1$ receptor signaling [23]. Thus, ECs partake in the control of homeostatic feed intake, whereas their action in the limbic system induces hedonic feed intake [4,24]. In the hypothalamus, ECs exert neuromodulatory effects by activating major orexigenic neurons located either in the arcuate nucleus (ARC), e.g., neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons, or in the lateral hypothalamus (LH), e.g., orexin-A (OX-A) neurons [9]. The pharmacological stimulation of $CB_1$ receptors augments hypothalamic NPY release [25], presumably through EC-mediated disinhibition via $CB_1$-containing axons innervating AgRP/NPY neurons [26]. Furthermore, EC-mediated $CB_1$ activation has been shown to enhance OX-A activity in the LH of mice [27], and a $CB_1$ and OX receptor heterodimerization has been demonstrated in various cell lines [28–30] as well as in the LH of mice [27].

As proposed by Allen et al. [31], cows provide an ideal animal model to study the regulation of food intake in species with continuous nutrient supply at different physiological stages [32]. In addition, cows allow frequent blood sampling with almost no or minimal disruption of food intake behavior. However, so far, little is known about the cow as an animal model for endocannabinoid research and about the involvement of the ECS and the impact of the EC tone in the regulation of food intake in ruminants. In a few pioneer studies, the relationship between the expression of genes related to the ECS or plasma EC concentrations and the energy status of cows have been investigated [33–35]. The baseline EC tone and ECS gene expression in cows has been investigated in bovine liver [33], subcutaneous adipose tissue [34] and the hypothalamus [35]. Specifically, feeding a ration exceeding energetic requirements increased the hepatic expression of $CB_2$ [33], and high postpartum weight loss and lipolysis increased the AEA and 2-AG content and expression of $CB_1$ and $CB_2$ in adipose tissue in cows [34]. Furthermore, Kuhla et al. [35] showed that increased plasma AEA and 2-AG concentrations postpartum are directly associated with the level of food intake in cows postpartum. However, so far, no study testing the cause–effect relationship between ECs and food intake has been performed in cows. Therefore, the objectives of this pilot study were to evaluate the effects of intraperitoneal (i.p.) AEA and 2-AG administration on systemically available EC concentrations, food intake and hypothalamic orexigenic signaling, and to further investigate how the circulatory EC tone changes in response to short-term energy deprivation.

## 2. Materials and Methods

### *2.1. Animals and Experimental Design*

All animal experiments were conducted in accordance with the ARRIVE guidelines ( https://arriveguidelines.org; accessed on 1 September 2018), the German Animal Welfare Act and approved by the ethical committee of the State Mecklenburg—Western Pomerania, Germany (Registration No. LALLF M-V 7221.3-1.1-041/18).

Experiment 1: Twenty non-pregnant Simmental cows from 1st to 10th lactation (>120 days in milk, DIM) with a mean body weight (BW) of 747 ± 15 kg and an average milk yield of 15.6 ± 1.1 L per day entered the experimental trial in five blocks of four. Each block consisted of a 20-day adaptation period followed by two 4-week feeding periods, each offering a different diet (Figure S1). Diets were fed in a cross over design, and the first 20 days of the second feeding period was considered the washout period. Diets were offered as total mixed ration (TMR, Table S1) and were based either on grass silage (GS) or corn silage (CS). Cows were housed in an experimental free-ranging barn at the Institute for Farm Animal Biology, Dummerstorf, Germany, and fed at 05:00 a.m. and again at 05:00 p.m. for ad libitum intake. Access to food was restricted between 05:00 a.m. and 08:00 a.m. to ensure a comparable start of food intake at 08:00 a.m. Cows were milked twice daily (05:30 a.m. and 05:30 p.m.) and milk yield from the evening and following morning milking was summarized as the daily milk yield. The BW was recorded after each

milking to calculate the mean metabolic BW ($BW^{0.75}$) as the weekly mean. After balancing for $BW^{0.75}$, age, milk yield and DIM, animals were allocated to three groups. The two EC treatment groups were injected either with 2.5 µg 2-AG/kg BW ($n$ = 6) or 5 µg AEA/kg BW ($n$ = 7), purchased from Tocris (Bioscience, Bristol, UK). Doses in the low microgram/kg range were chosen because they were most effective in food intake response studies in rodents [20,22] and exert no unwanted cannabimimetic side effects [36,37]. The ECs were pre-dissolved in ethanol and diluted in 8 mL of 0.9% NaCl right before injection. The control group was injected with 8 mL of a sterile 0.9% NaCl solution ($n$ = 7). Treatments were administered daily during the last 8 days of each feeding period at 08:00 a.m. via i.p. injections (1.20 mm × 80 mm, SUPRA, Vivomed, Geislingen, Germany) into the right paralumbar fossa. The i.p. route of administration was chosen to allow absorption from the peritoneal cavity into the systemic circulation, as well as to activate the gastrointestinal ECS and vagal afferents of the gut–brain axis [12,13]. From day 6 of each treatment period onwards, cows were kept in tie-stalls to prevent social interaction during food intake. Cows were fitted with a jugular vein catheter (Cavafix Certo Splittocan 338, B. Braun Melsungen AG, Melsungen, Germany) connected to a 4 m extension line to allow blood sampling without interference of intake behavior. On day 8 of each treatment period, blood samples were collected in EDTA-containing tubes 1 h before, 2.5 and 5.5 h after i.p. administrations. Cows kept in tie-stalls were milked at 06:00 a.m. and 04:00 p.m. Troughs were emptied at 06:00 a.m. and cows were given access to food at 08:00 a.m. and 05:00 p.m. Food intake was recorded as disappearance from trough every 6 min by an electronic registration device (PAARI, Erfurt, Germany). The food dry matter (DM) content was determined, and dry matter intake (DMI) was calculated. At the end of the second feeding period (day 9 of the treatment period), an additional i.p. injection was given at 07:00 a.m., approximately 1 h before slaughter of the control and approximately 2 to 3 h before slaughter of the EC-treated animals. After transfer to the institute's slaughterhouse, located approximately 300 m from the stall, cows were anesthetized by captive bolt stunning and exsanguinated, and the hypothalamus was isolated within 20 min post mortem.

Experiment 2: Seven lactating, non-pregnant Simmental cows from 1st to 6th lactation (>120 DIM) with a mean BW of 778 ± 25 kg and an average milk yield of 16.7 ± 2.5 L per day were used to study the response of plasma EC concentrations relative to energy deprivation. The cows were kept in a free-ranging barn and fed a CS-based TMR (Table S1), as described above. For food deprivation, troughs were emptied and access to the Roughage Intake Control system (RIC, Insentec B. V., Marknesse, The Netherlands) was blocked between 07:00 a.m. and 02:00 p.m. At 02:00 p.m., troughs were refilled, and cows were given back their access to ad libitum intake. Immediately before the start of the food deprivation period, at the end of 7 h food deprivation, and 17 h after refeeding, a blood sample was taken from the tail vein and collected in EDTA-containing tubes.

### *2.2. Analyses of Plasma Endocannabinoids and Metabolites*

For the analysis of plasma EC concentrations in experiment 1 and 2, blood samples were immediately placed on ice, centrifuged at 1570× $g$ for 20 min at 4 °C, and stored at −80 °C. Samples were analyzed for AEA and 2-AG concentrations by the Research Core Unit Metabolomics at the Hannover Medical School using the cross-validated method as described recently [38]. Briefly, analyses were carried out using a Waters ACQUITY UPLC-MS/MS system with a tandem quadruple mass spectrometer XEVO TQ MS (Waters, Milford, MA, USA), using a Waters ACQUITY BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm particle size) for separation of analytes. Plasma-free, non-esterified fatty acid (NEFA) and glucose concentrations were analyzed spectrophotometrically at a semi-automatic analyzer (ABX Pentra 400, HORIBA Medical, Kyoto, Japan) using the respective NEFA-HR 91797 (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) and Glucose HK CP A11A01667 (HORIBA) kits.

### *2.3. Brain Sampling, mRNA Isolation and RT-qPCR*

The hypothalamus obtained in experiment 1 was isolated by two frontal sections at the optic chiasm and the mammillary bodies. The hypothalamic region was isolated by two diagonal sections from the fornix to the most ventro-lateral site of the optic tract. The right hypothalamic hemisphere was transferred to a 4% Formafix solution (Grimm Logistik GmbH, Torgelow, Germany) and stored at 4 °C for at least 4 weeks. From the left hypothalamus, the paraventricular nucleus (PVN) and arcuate nucleus (ARC) were isolated, immediately snap frozen in liquid nitrogen and stored at −80 °C until further analysis. Briefly, tissue samples were ground under liquid nitrogen and total RNA was extracted from 19 to 43 mg tissue powder using an innuPREP RNA Mini Kit 2.0. Residual DNA was removed with an innuPREP DNase I Digest Kit (Analytik Jena AG, Jena, Germany). Integrity and the quality of the obtained mRNA was confirmed after electrophoresis on agarose gel stained with ethidium bromide (Carl Roth GmbH) and by measurement of the optical density 260:280 ratio. RNA concentrations were determined spectrophotometrically on a NanoPhotometer (Implen GmbH, Munich, Germany). Subsequently, 750 ng of total RNA was reverse-transcribed with a SensiFAST cDNA Synthesis Kit (Bioline, London, UK). Real-time quantitative PCR was performed on a LightCycler 2.0 (Roche, Basel, Switzerland) with 2 µL of cDNA using the SensiFAST SYBR No-ROX Kit (Bioline). Each cDNA sample was analyzed in duplicate. The efficiency of amplification was calculated using LinRegPCR software, version 2014.4 (Academic Medical Centre, Amsterdam, The Netherlands), yielding efficiency values between 1.81 and 1.92 (Table S2). Data were quantified using qbasePlus software (Biogazelle, Gent, Belgium) and normalized to the most stable genes, eukaryotic translation initiation factor 3 subunit K (*EIE3K*) and peptidylprolyl isomerase A (*PPIA*). Primers used for the analysis of orexigenes and genes related to the ECS are shown in Table S2. Due to the limited amount of mRNA obtained from the left ARC of some animals, expression analysis for *NPY* and *PLAAT5* could only be performed for NaCl ($n$ = 7), AEA ($n$ = 7), 2-AG ($n$ = 4), and NaCl ($n$ = 5), AEA ($n$ = 6), 2-AG ($n$ = 6), respectively.

### *2.4. Immunohistochemistry (IHC)*

Tissue of the right hypothalamic hemisphere was dehydrated in a series of ethanol solutions of increasing concentrations up to 100%, embedded in paraffin, and subsequently cut on a microtome (Leica RM2145, Wetzlar, Germany) in 4 µm sections. Slices were mounted on Dako Flex IHC slides and dried in an incubator for one hour at 60 °C. For the analysis of $CB_1$-positive cells, slices were pre-treated to block peroxidases as recently described [35]. Slices were incubated overnight with a polyclonal rabbit IgG against $CB_1$ (Abcam, Cambridge, UK; ab23703; 1:500) followed by 1 h incubation with a goat anti-rabbit HRP antibody (Agilent Dako, Santa Clara, CA USA; 1:100) at room temperature. Immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB) precipitation using the LSAB2 System-HRP kit (Dako; K0675). Slices were counterstained with hemalaun to visualize cell nuclei. Negative controls were incubated omitting the primary antibody. No unspecific binding of the secondary antibody was detected. Slices were visualized with a BX51 light microscope (OLYMPUS, Hamburg, Germany) equipped with 20× magnification and an SC50 camera (OLYMPUS) with the cellSens Standard software (OLYMPUS). Five to ten images of the PVN were taken per animal. For blinded image analysis, image colors were de-convoluted using the IHC profiler of the ImageJ software (National Institutes of Health, Bethesda, MD, USA) [39]. The pixel intensities of DAB images were analyzed using the color threshold method of ImageJ with brightness ranging from 229 to 245.

For immunofluorescence-based co-localization studies, slices were incubated with a polyclonal guinea pig anti-AgRP antibody (Abcam; ab228495, 1:200) together with a rabbit-anti-cFos antibody (Abcam; ab99515, 1:100). After overnight incubation, the slices were washed 3 times in phosphate-buffered saline (pH 7.4) for 10 min, followed by incubation with secondary fluorescence-labeled antibodies: goat-anti-guinea pig Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA USA; 1:500) and goat-anti-rabbit DyLight 594 (Thermo Fisher Scientific; dilution 1:100) for 1 h at room temperature. The slices were

counterstained with DAPI (AppliChem GmbH, Germany) for 15 min. For the negative control, slices were incubated omitting the primary antibody. No unspecific binding of the secondary antibody was detected. For the co-localization between Orexin A and cFos, primary sheep-anti-Orexin A antibody (NB100-65204; Bio-Techne, Minneapolis, MN, USA; 1:200) and secondary donkey-anti-sheep antibody (Alexa Fluor 488, Thermo Fisher Scientific; 1:100) were applied by using the same protocol as described above. Slices were analyzed with an Axioskop 40 FL fluorescence microscope (ZEISS, Oberkochen, Germany) at 20× magnification and images were acquired with an AxioCam MRc5 camera, controlled by ZEN 2 lite software (ZEISS). For AgRP analysis, four to five images per animal were taken and analyzed blinded using an automated quantitative analysis of ImageJ (NIH). Briefly, images were converted to 16 bit greyscale and the threshold was manually adjusted to only highlight and select the cell structures. After obtaining the binary image, pixels were counted according to the following criteria: size (pixel^2): 0.01-Infinity and circularity: 0.00–1.00. For Orexin A analysis, two to four images were taken per animal, but due to technical biases, three slices could not be evaluated, resulting in the analysis of NaCl ($n$ = 6), AEA ($n$ = 6) and 2-AG ($n$ = 5). Image analysis was performed blinded using the Cell Counter plugin of the ImageJ software (NIH) for manual cell counting.

### *2.5. Statistical Analyses*

Statistical analyses were performed using the SAS software for Windows, version 9.4 (SAS Institute Inc., Cary, NC, USA). Data were analyzed with repeated measurement analyses of variance using the MIXED procedure in SAS/STAT software. DMI/$BW^{0.75}$ and cumulative DMI/$BW^{0.75}$ were evaluated on day 8 in hourly intervals between 1 and 6 h post-injection. The respective data were statistically analyzed in hourly intervals with the fixed effects treatment (NaCl, AEA and 2-AG), diet (GS and CS) and time (interval), with diet and time as repeated variables. Plasma AEA and 2-AG concentrations were also analyzed using repeated measures ANOVA with fixed effects for treatment, diet and time of sampling, with diet and time as repeated variables. Data obtained from IHC and mRNA expression analyses were first analyzed using ANOVA; for mRNA expression analysis, the model contained the fixed effects treatment and diet, and for IHC analysis, the model contained the fixed effects treatment, diet and image, with image as the repeated variable. Because the effect of diet was found not significant, IHC and mRNA data were subsequently analyzed using ANOVA, including the fixed effect treatment (for mRNA) or the fixed effects treatment, and image, with image as the repeated variable (for IHC analysis). Data were tested for normal distribution using the Shapiro–Wilk test and transformed using Johnson transformation prior to testing. To evaluate the EC, NEFA and glucose concentrations in experiment 2, the model contained the fixed effect time, with time as the repeated variable. The calculation of Pearson correlation between NEFA, glucose and EC concentrations was performed by using the CORR procedure of SAS. Least square means (LSMs) and their standard error (SE) were calculated and pairwise tested for each fixed effect in the models described above by using the Tukey–Kramer procedure for pairwise multiple comparisons. Effects and differences at $p < 0.05$ were considered significant.

## 3. Results

### *3.1. Experiment 1*

#### 3.1.1. Body Weight, Milk Yield and Intake

Body weight, $BW^{0.75}$ and milk yield did not differ between groups (data not shown). The DMI normalized to metabolic body weight (DMI/$BW^{0.75}$) was assessed on day 8 for 6 h after administrations and was found to be different between the treatment groups ($F$(2,36) = 7.73, $p < 0.01$) and over time ($F$(6,106) = 73.3, $p < 0.001$) but not between diets ($F$(1,30) = 0.03, $p$ = 0.87) (Figure 1a). More specifically, within the first h after injection, i.p. 2-AG administration resulted in a 41.5 and 88.8% higher DMI/$BW^{0.75}$ compared to the control group ($p < 0.01$) on the GS and CS diet, respectively. During the same time, i.p. AEA compared to the control administration increased DMI/$BW^{0.75}$ only by 11.8% on the GS diet,

but by 70.7% on the CS diet ($p < 0.05$). Between 2 and 6 h post-treatment, DMI/BW$^{0.75}$ was not significantly different between groups. Likewise, cumulative DMI/BW$^{0.75}$ was different between treatment groups ($F(2,28) = 4.10$, $p < 0.05$) but not between diets ($F(1,39) = 1.93$, $p = 0.17$). Cumulative DMI/BW$^{0.75}$ increased over time ($F(6,88) = 221.09$, $p < 0.001$), resulting in a 52.5 and 42.0% higher intake 6 h after i.p. 2-AG administration compared to the control group on the GS and CS diet, respectively ($p < 0.05$, Figure 1b). The i.p. AEA administration increased the 6 h cumulative DMI/BW$^{0.75}$ by 32.2 and 42.8% compared to the control group on the GS and CS diet, respectively ($p < 0.05$).

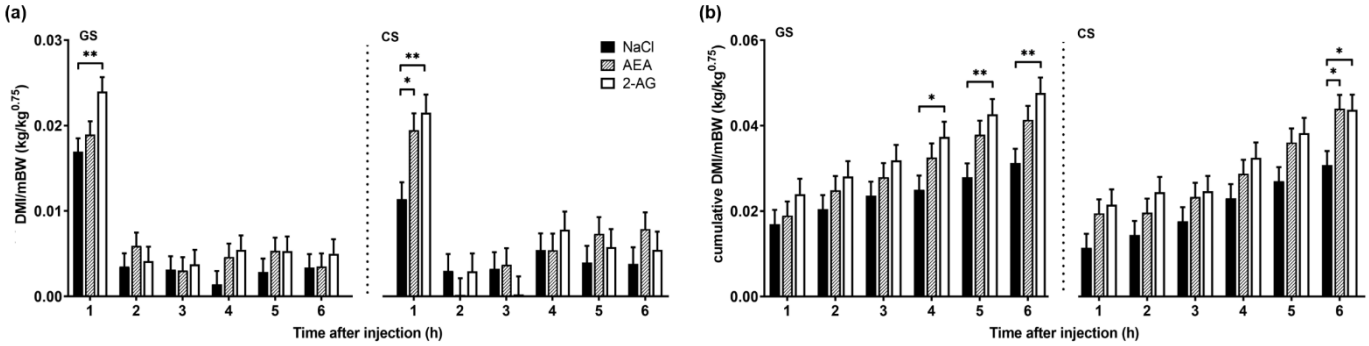


**Figure 1.** Dry matter intake per metabolic body weight DMI/BW$^{0.75}$ (**a**) and cumulative DMI/BW$^{0.75}$ (**b**) on day 8, 1 to 6 h after intraperitoneal administration with NaCl ($n = 7$), AEA ($n = 7$) or 2-AG ($n = 6$). Cows were fed ad libitum a grass silage (GS) and corn silage (CS)-based diet. Data are shown as LSM ± SE. Treatment differences are indicated by * $p < 0.05$ and ** $p < 0.01$ (Tukey–Kramer). ANOVA results are presented in Table S3.

#### 3.1.2. Plasma Endocannabinoid Concentrations

We next examined whether i.p. EC injection affected plasma AEA and 2-AG concentrations. Overall, plasma AEA concentrations were not different between treatment groups ($F(2,29) = 0.96$, $p = 0.40$). However, plasma AEA levels changed over time in the AEA-treated group ($F(2,40) = 3.41$, $p < 0.05$). Relative to the pre-injection level, the i.p. administration of 5 µg/kg AEA increased plasma AEA concentrations 2.5 h post-injection to 136.7 and 156.9% on the GS and CS diet, respectively ($p < 0.001$; Figure 2a). This increase was significantly higher compared to the control and 2-AG group on both diets ($p < 0.05$). From 2.5 to 5.5 h after AEA injection, plasma AEA concentrations declined to baseline levels on the GS ($p < 0.001$) and CS ($p < 0.01$) diet. By contrast, plasma AEA concentrations remained constant in the control and 2-AG group. Furthermore, there was no change in plasma 2-AG concentrations after AEA, 2-AG or NaCl treatments ($F(2,27) = 1.14$, $p = 0.34$) or over time ($F(2,37) = 1.44$, $p = 0.25$) (Figure 2b). The diets had no effect on either AEA or 2-AG levels ($F(1,37) = 0.21$, $p = 0.65$) ($F(1,21) = 2.41$, $p = 0.14$), respectively.

#### 3.1.3. Transcription of Hypothalamic Genes

Analysis of mRNA abundance of the orexigenic neuropeptides AgRP and NPY in the ARC tended to be different between treatment groups ($F(2,17) = 3.13$, $p = 0.07$) ($F(2,15) = 2.81$, $p = 0.09$), respectively (Table 1). More specifically, the abundance of *AGRP* was approximately 70% and the abundance of *NPY* 78% lower in AEA compared to the control and 2-AG group, respectively. However, these differences were not observed in the PVN. The mRNA abundances of genes involved in the ECS (*CB1*, *DAGLA*, *FAAH*, *NAPLD* and *PLAAT5*) of the ARC and PVN were not different between treatment groups (Table 1).

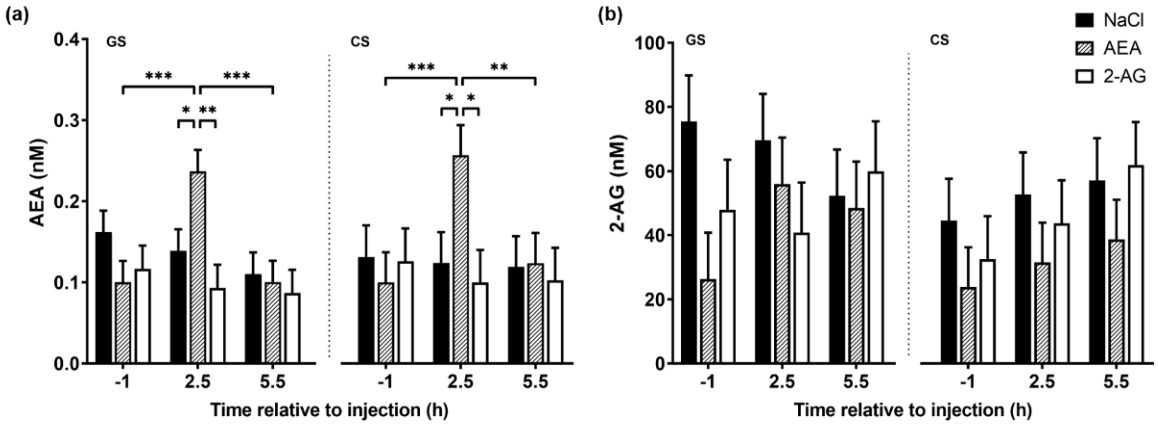


**Figure 2.** Plasma endocannabinoid concentrations on day 8, 1 h before, 2.5 h and 5.5 h after intraperitoneal administration with NaCl ($n$ = 7), AEA ($n$ = 7) or 2-AG ($n$ = 6). Cows were fed ad libitum a grass silage (GS) and corn silage (CS)-based diet. Data for (**a**) AEA (nM) and (**b**) 2-AG (nM) are shown as LSM ± SE. Treatment and time differences are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Tukey–Kramer). ANOVA results are presented in Table S3.

**Table 1.** Relative mRNA abundances (LSM ± SE) of orexigenic neuropeptides and genes related to the ECS of the arcuate nucleus (ARC) and paraventricular nucleus (PVN). Samples were obtained from cows treated with NaCl ($n$ = 7), AEA ($n$ = 7) or 2-AG ($n$ = 6) for 9 days.

| Gene | NaCl | AEA | 2-AG | ANOVA |
|---|---|---|---|---|
| ARC | | | | |
| *AGRP* | 1.72 ± 0.46 | 0.55 ± 0.04 | 2.03 ± 0.87 | $F(2,17) = 3.13$, $p = 0.07$ |
| *CNR1* | 1.03 ± 0.16 | 1.06 ± 0.17 | 1.11 ± 0.14 | $F(2,17) = 0.06$, $p = 0.95$ |
| *DAGLA* | 1.00 ± 0.09 | 0.98 ± 0.09 | 1.07 ± 0.07 | $F(2,17) = 0.29$, $p = 0.75$ |
| *FAAH* | 1.07 ± 0.18 | 0.98 ± 0.10 | 1.12 ± 0.07 | $F(2,17) = 0.31$, $p = 0.73$ |
| *NAPELD* | 1.05 ± 0.03 | 0.95 ± 0.11 | 1.07 ± 0.11 | $F(2,17) = 0.53$, $p = 0.60$ |
| *NPY* * | 1.84 ± 0.70 | 0.42 ± 0.11 | 1.91 ± 0.48 | $F(2,15) = 2.81$, $p = 0.09$ |
| *PLAAT5* * | 1.16 ± 0.45 | 1.43 ± 0.42 | 1.05 ± 0.23 | $F(2,14) = 0.39$, $p = 0.68$ |
| PVN | | | | |
| *AGRP* | 1.17 ± 0.17 | 1.15 ± 0.38 | 1.48 ± 0.47 | $F(2,17) = 0.24$, $p = 0.79$ |
| *CNR1* | 0.91 ± 0.07 | 1.07 ± 0.24 | 1.37 ± 0.24 | $F(2,17) = 1.12$, $p = 0.35$ |
| *DAGLA* | 0.99 ± 0.06 | 1.06 ± 0.10 | 1.00 ± 0.14 | $F(2,17) = 0.16$, $p = 0.85$ |
| *FAAH* | 0.99 ± 0.10 | 1.00 ± 0.07 | 1.08 ± 0.09 | $F(2,17) = 0.29$, $p = 0.75$ |
| *NAPELD* | 1.01 ± 0.09 | 1.06 ± 0.09 | 1.00 ± 0.08 | $F(2,17) = 0.12$, $p = 0.89$ |
| *PLAAT5* | 1.38 ± 0.24 | 1.26 ± 0.46 | 1.65 ± 0.66 | $F(2,17) = 0.18$, $p = 0.84$ |

* Because of the limited amount of ARC mRNA, evaluable results were limited to NaCl ($n$ = 7), AEA ($n$ = 7), 2-AG ($n$ = 4) and NaCl ($n$ = 5), AEA ($n$ = 6), 2-AG ($n$ = 6), respectively. AGRP, agouti-related neuropeptide; CNR1, cannabinoid receptor 1; DAGLA, diacylglycerol lipase alpha; FAAH, fatty acid amide hydrolase; NAPEPLD, N-acyl phosphatidylethanolamine phospholipase D; NPY, neuropeptide Y; PLAAT5, phospholipase A and acyltransferase 5.

### 3.1.4. Immunohistochemistry

When analyzed on the protein level, $CB_1$ immunoreactivity of neurons located in the PVN was not different between groups ($F(2,15) = 0.15$, $p = 0.86$) (Figure 3). Despite the strong immunoreactivity of AgRP neurons in the ARC, both the number of AgRP cells ($F(2,17) = 0.25$, $p = 0.80$) and the number of cFos-positive AgRP cells ($F(2,17) = 0.51$, $p = 0.61$) were not statistically different between treatment groups (Figure 4). In addition, the resulting percentage of activated AgRP cells per total number of AgRP cells was also not different ($F(2,15) = 2.11$, $p = 0.16$). Likewise, there were no differences between treatment groups for orexin-A ($F(2,14) = 0.58$, $p = 0.57$), cFos immunofluorescence staining ($F(2,14) = 0.61$, $p = 0.56$) and the resulting percentage of activated orexin-A cells per total number of orexin-A cells in the LH ($F(2,13) = 0.45$, $p = 0.65$) (Figure 5).

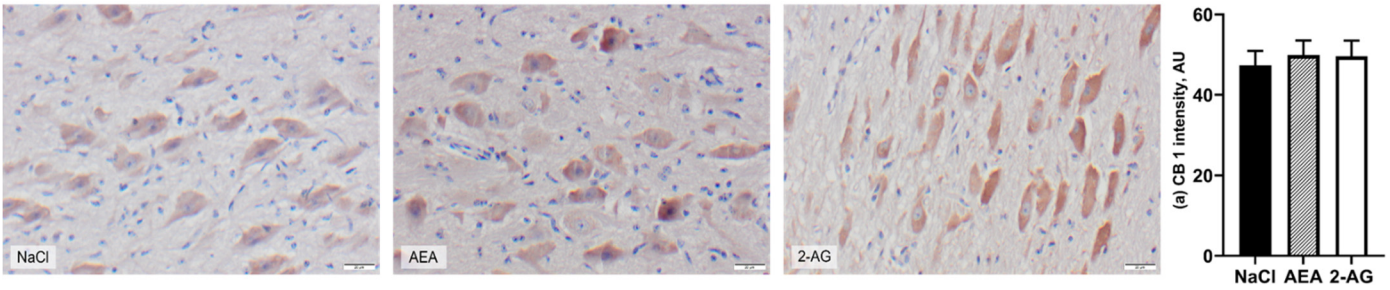


**Figure 3.** Representative images of DAB-stained CB1-expressing neurons in the periventricular nucleus (PVN) of cows after intraperitoneal administration with NaCl ($n$ = 7), AEA ($n$ = 7) or 2-AG ($n$ = 6) for 9 days. CB1 immunoreactivity was visualized with DAB (arbitrary units, AU) and nuclei were stained with hemalaun (blue). Scale bar within images indicates 20 µm. CB1 intensities (**a**) are shown as LSM ± SE with $F(2,15) = 0.15$, $p = 0.86$ (ANOVA).

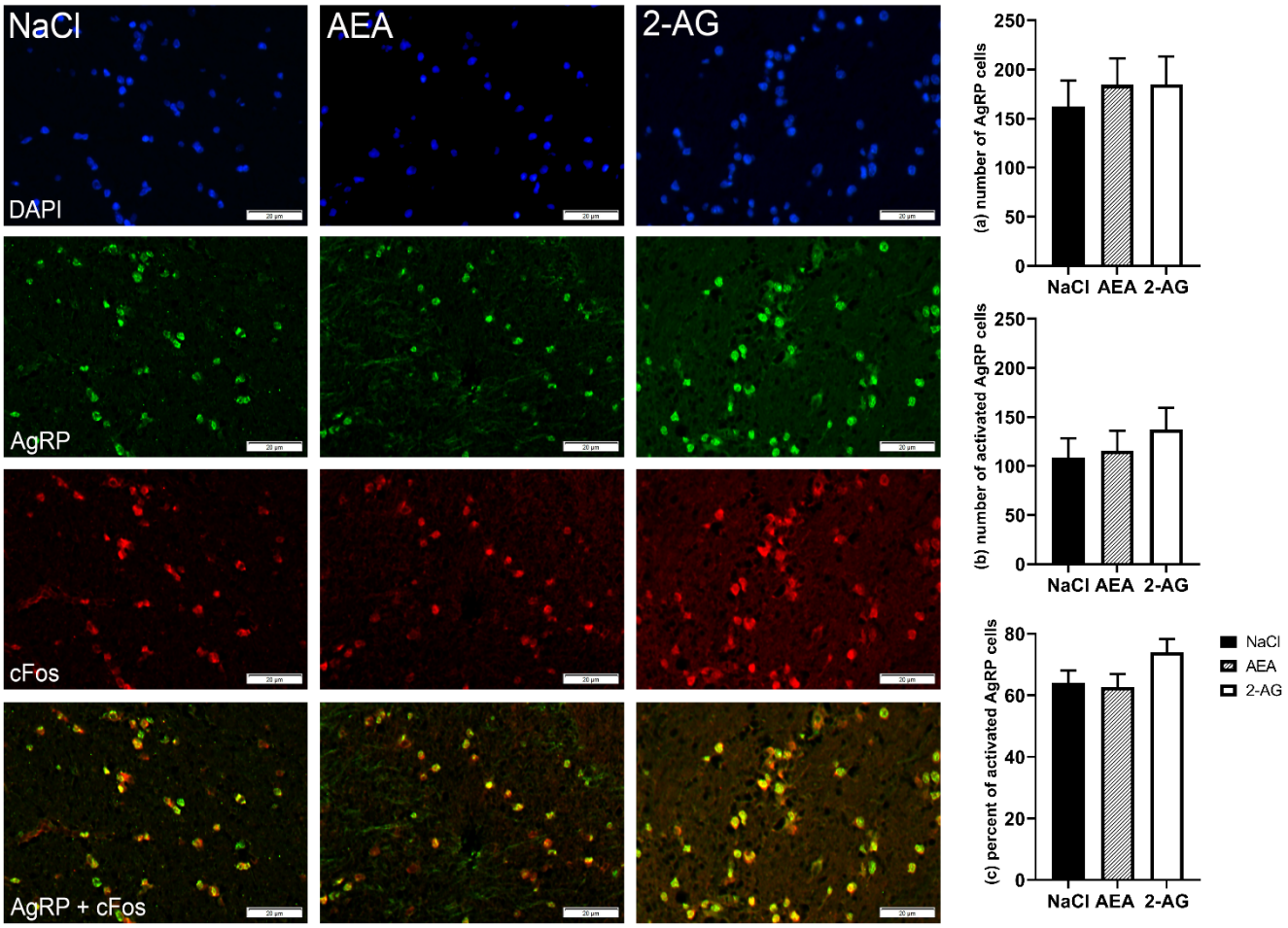


**Figure 4.** Immunofluorescence staining and representative images of the arcuate nucleus (ARC) of cows after intraperitoneal administration with NaCl ($n$ = 7), AEA ($n$ = 7) or 2-AG ($n$ = 6) for 9 days. DAPI-stained nuclei (blue), AgRP neurons (green), cFos (red) and co-localization (merged image). Scale bar within images indicates 20 µm. Number of AgRP neurons (**a**), number of cFos-positive AgRPcells (**b**) and the percentage of activated AgRP cells per total AgRP cells (**c**). Data are shown as LSM ± SE with (**a**) $F(2,17) = 0.25$, $p = 0.80$, (**b**) $F(2,17) = 0.51$, $p = 0.61$, (**c**) $F(2,15) = 2.11$, $p = 0.16$ (ANOVA).

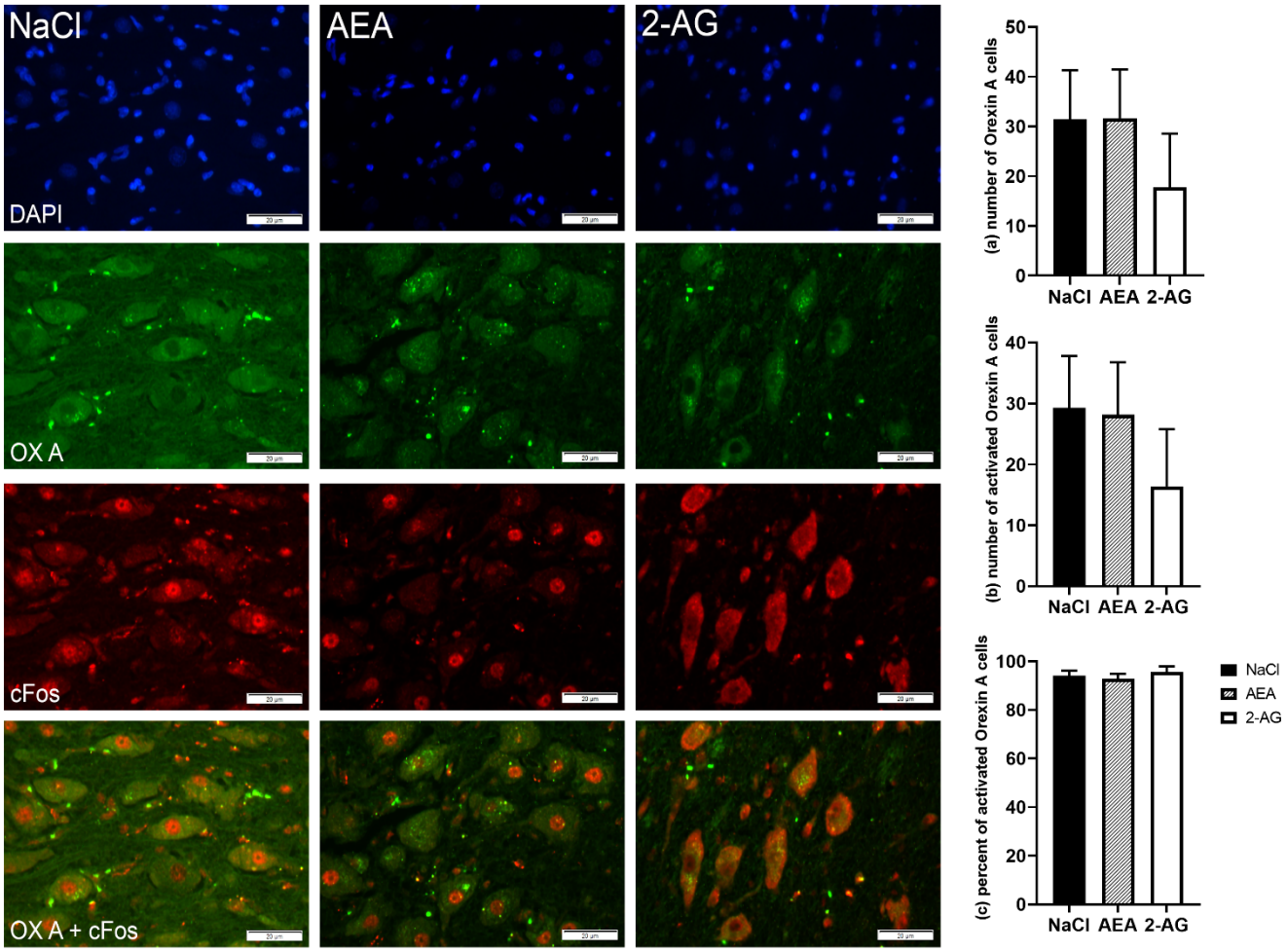


**Figure 5.** Immunofluorescence staining and representative images of the lateral hypothalamus (LH) of cows after intraperitoneal administration with NaCl ($n$ = 6), AEA ($n$ = 6) or 2-AG ($n$ = 5) for 9 days. DAPI-stained nuclei (blue), orexin-A neurons (green), cFos (red) and co-localization (merged image). Scale bar within images indicates 20 µm. Number of orexin-A neurons (**a**), number of cFos-positive orexin-A cells (**b**) and the percentage of activated orexin-A cells per total orexin-A cells (**c**). Data are shown as LSM ± SE with (**a**) $F(2,14) = 0.58$, $p = 0.57$, (**b**) $F(2,14) = 0.61$, $p = 0.56$, (**c**) $F(2,13) = 0.45$, $p = 0.65$ (ANOVA).

### *3.2. Experiment 2*

#### Food Deprivation

Plasma 2-AG concentrations increased 7 h after food withdrawal relative to the basal level ($p < 0.05$, Table 2). This increase was accompanied by increased plasma NEFA ($p < 0.01$) and decreased plasma glucose concentrations ($p < 0.01$). After refeeding, plasma 2-AG ($p < 0.05$), but not plasma NEFA and glucose concentrations, returned to baseline levels. Pearson correlation analysis including samples from all time points investigated reveal that 2-AG and NEFA concentrations were positively correlated ($r = 0.63$; $p < 0.01$) and 2-AG and glucose concentrations were negatively correlated ($r = -0.50$; $p < 0.05$). In addition, plasma AEA concentrations did not change in response to food deprivation.

**Table 2.** Plasma endocannabinoid (AEA, 2-AG), NEFA and glucose concentrations of 7 cows with ad libitum intake, after 7 h of food deprivation and 17 h after refeeding. Cows were offered a corn silage-based diet. Data are shown as LSM + SE.

| | Ad Libitum | Food Deprivation | Refeeding | ANOVA |
|---|---|---|---|---|
| AEA (nM) | 0.26 $^{a}$ ± 0.02 | 0.29 $^{a}$ ± 0.02 | 0.27 $^{a}$ ± 0.02 | $F(2,12) = 0.69$, $p = 0.52$ |
| 2-AG (nM) | 3.60 $^{a}$ ± 0.49 | 4.73 $^{b}$ ± 0.49 | 3.63 $^{a}$ ± 0.49 | $F(2,12) = 6.17$, $p < 0.05$ |
| NEFA (µmol/L) | 162.71 $^{a}$ ± 40.28 | 237.14 $^{b}$ ± 40.28 | 189.29 $^{ab}$ ± 40.28 | $F(2,12) = 7.46$, $p < 0.01$ |
| Glucose (mmol/L) | 4.06 $^{a}$ ± 0.13 | 3.65 $^{b}$ ± 0.13 | 3.73 $^{b}$ ± 0.13 | $F(2,12) = 8.38$, $p < 0.01$ |

$^{a,b}$ Different superscript letters within one row indicate significance differences ($p < 0.05$, Tukey–Kramer).

## 4. Discussion

### *4.1. Effect of Endocannabinoids on Food Intake*

The present study demonstrates that systemically administered 2-AG and AEA increase food intake in cows in the short term. Specifically, the i.p. administration of 2.5 µg 2-AG/kg BW (on GS and CS) as well as 5 µg AEA/kg BW (on CS) resulted in a higher increase in food intake during the first hour after injection compared to the control group. Although the differences in food intake were not apparent when analyzed for the subsequent hourly intervals, cumulative food intake of 2-AG animals was greater 4 to 6 h on the GS, and of AEA-treated cows 6 h post-injection on the CS diet. Despite repeated daily i.p. administrations, food intake of cows did not increase in the long term, e.g., over 8 days [40]. In contrast, daily i.p. administrations of 1 µg AEA and 2-AG per kg BW increased food intake in mice over 7 and 14 days [20,22]. However, the administration of a single EC dose into the brain of mice only increased food intake for 1 h post-injection [41]. The observed short-term effect of EC on food intake might be due to the short half-life of AEA and 2-AG lasting only for a few minutes in mice [17,18].

The EC-mediated control of food intake is complex, and $CB_1$ activation has been shown to promote food intake at various levels in the CNS [42]. For example, peripheral [43] and central [44] $CB_1$ activation influences the motivation to consume palatable foods and promotes food intake through increased odor detection in the main olfactory bulb of mice [45]. Although our study did not aim to investigate the interaction between ECs and palatability, it is tempting to speculate that stronger impact of AEA and 2-AG on the CS compared to GS intake is due to the higher increase in palatability of the CS diet. Because the CS and GS rations did not have the same energy content, it may also be the case that AEA and 2-AG administrations particularly support the ingestion of high energy density diets. Therefore, it would be of great interest to investigate the effect of ECs on taste and energy content preferences as wells as hedonic aspects of food intake in ruminants in the near future.

### *4.2. Effect of Endocannabinoids on Plasma AEA and 2-AG Concentration*

In addition to the direct effect on splanchnic EC receptors and vagal afferent neurons [10,12,13], i.p.-administered ECs can be rapidly (seconds to minutes) absorbed from the peritoneal cavity into systemic circulation due to their low molecular weight and high lipophilicity [46]. The high lipophilicity allows EC to be rapidly removed from the circulation and distributed into tissues. Willoughby et al. [17] demonstrated that radiolabeled AEA (50 mg/kg) was detectable in the brain of mice as early as 1 min after i.v. injection. In our study, we did not sample plasma before 2.5 h post-administration and thus may have missed the time of highest accumulation of ECs in the circulation. However, the time point of plasma sampling 2.5 h post-administration was chosen to coincide with the collection of hypothalamic tissue. Plasma AEA concentrations were significantly increased 2.5 h post-injection of AEA, whereas AEA concentrations remained stable after 2-AG or NaCl administration. This finding suggests that i.p.-administered AEA accumulates in

the circulation of cows and has a longer half-life than only a few minutes, as found in rodents [17]. Furthermore, Kozak et al. [47] demonstrated a much longer half-life of 2-AG when incubated with human plasma as compared to rat plasma, suggesting that the rat is not a suitable model to study the biological activities of ECs in humans. Our findings imply a longer half-life, at least for AEA, but whether the cow provides a more adequate model requires further investigation in the future. Moreover, our findings are in agreement with the results from Oddi et al. [15], demonstrating that AEA is transiently stored in lipid droplets in adipose tissue and can potentially re-enter the circulation with a timely delay. Previous studies also showed a biphasic pattern in radioactivity enrichment in plasma and various tissues after the i.v. administration of tracer-labeled AEA in mice [17], further supporting the idea of a longer-lasting exchange of AEA between the circulation and tissues. However, the bioavailability of AEA distinguishes from the bioavailability of 2-AG, because we did not observe changes in plasma 2-AG concentrations 2.5 or 5.5 h after i.p. 2-AG administration. Either 2-AG is stored longer in tissues or, more likely, has a shorter half-life than AEA. A tracer study with higher frequency sampling would be needed to determine the exact half-life for AEA and 2-AG.

### *4.3. Effect of Endocannabinoids on Hypothalamic Orexigenic Circuits*

By binding to $CB_1$ receptors expressed by vagal afferent neurons [10,12,13,48] and rapidly crossing the blood–brain barrier [49], peripherally administered ECs can modulate the release and action of neurotransmitters [19]. To elucidate these neuromodulatory effects, for the first time, we investigated how peripherally administered AEA and 2-AG affect hypothalamic orexigenic neuropeptides and genes related to the ECS. Central $CB_1$ receptors play an essential role in the neuronal network of food intake and appetite stimulation [42]. In fact, Soria-Gómez et al. [50] demonstrated that AEA administration into the nucleus accumbens shell (NAcS) of rats did not only increase food intake but also cFos immunoreactivity in hypothalamic nuclei. Furthermore, presynaptic $CB_1$ activation led to a suppressed inhibitory tone in perifornical LH neurons [51]. By using 3D reconstruction of serial ARC sections of mice, Morozov et al. [26] demonstrated that $CB_1$-positive synapses innervate NPY/AgRP neurons, further strengthening an EC-mediated retrograde disinhibition of these orexigenic neurons. Moreover, in rat hypothalamic explants, a direct effect of ECs on hypothalamic NPY has been shown [25]. Specifically, the pharmacological stimulation of $CB_1$ receptors with AEA and the synthetic agonist CP55940 increased hypothalamic NPY release. Based on these findings, we expected the activation of hypothalamic AgRP and NPY after enhancing the systemic EC tone. Conversely, AgRP immunoreactivity was not affected by either treatment, and *AGRP* and *NPY* mRNA abundances in the ARC of AEA-administered animals tended to be markedly lower than in the 2-AG and control groups. In addition, the expression of genes related to ECS and $CB_1$ immunoreactivity were not affected by i.p. AEA and 2-AG injection. Failure to detect increased $CB_1$ expression or activation of NPY/AgRP neurons might be due to time between i.p. injection and tissue gain of 2 to 3 h. Although AEA concentrations in the circulation were increased after 2.5 h, food intake did not differ in the second and third hour after AEA administration. This finding suggests that the activation of orexigenic neurons occurred presumably in the first hour after EC administration and could not be detected after 2 to 3 h. A collection of tissue samples 1 h after injection and during feeding would have been preferable; however, due to the necessary intermediary transport of the animals to the institute's slaughterhouse, this could not be achieved. Another reason might have been an increased stress level during the transport of the animals to the slaughterhouse, which may have suppressed the former EC-mediated activation of NPY/AgRP neurons. Nonetheless, NPY and AgRP mRNA expression in the ARC of AEA-treated cows was lower than in control animals, which could be a counter-regulatory mechanism reducing the preceded activation of NPY/AgRP neurons. The direct interaction between ECs and the OX system has been demonstrated in several studies, i.e., by the co-expression of $CB_1$ and OX receptors in various cell lines [28,29,52]. Furthermore, EC-mediated $CB_1$ activation enhanced OX-A

release by reducing the inhibition of respective neurons [27], and the inverse $CB_1$ agonist AM251 decreased the expression of OX-A in the hypothalamus of rats [53]. In our study, we did not detect an increase in OX-A immunoreactivity or the c-FOS activation of OX-A-expressing neurons despite coinciding increased circulatory AEA concentrations. Again, food intake was not different between treatment groups in the second and third hour after EC administration, and thus we can only speculate whether OX-A-expressing neurons were activated in the first hour after AEA administration when food intake was higher than in control animals or if transport stress to the slaughterhouse suppressed EC-induced OX-A activation. Another important aspect is that ECs interact with anorexic circuits. For example, $CB_1$ activation mediates the downregulation of POMC synthesis and $\alpha$-MSH release in the ARC of mice [54]. In the present study, we did not analyze the expression of anorexic neuropeptides, which, however, should be considered in future studies to better understand the EC-mediated regulation of food intake in the bovine brain. Furthermore, EC tone in the hypothalamus could not be analyzed herein because the brain was contaminated with blood after captive bolt stunning.

### *4.4. Effect of Food Deprivation on Plasma Endocannabinoid Concentrations*

A series of studies have investigated the effect of food deprivation on EC tone in the brain and small intestine of rodents [21,41,55]. Specifically, fasting resulted in increased 2-AG levels in the limbic forebrain, hypothalamus, and jejunal mucosa of rats [41,55]. Furthermore, fasting increased AEA levels in the small intestine of rats [21] and the blood of healthy, normoweight humans [56,57].

In the current study, plasma 2-AG significantly increased after 7 h of food deprivation and decreased to baseline levels after refeeding, whereas plasma AEA remained unchanged. The latter findings are consistent with the findings of Kirkham et al. [41], indicating that, as in rats, 2-AG in cows is most sensitive to short-term fasting and may be more involved in the immediate response to changes in energy supply by inducing appetite. Indeed, increased plasma 2-AG levels could originate from an increased 2-AG production in the small intestine [21,55] or from visceral adipose tissue [58]. However, our results are in contrast with findings in humans, where AEA levels increased with fasting [57], but no meal-related changes were observed for 2-AG [59]. Whether this indicates an inter species difference or more likely is due to time-dependent variations of EC levels in response to energy deficits [60] needs to be determined in future studies.

To confirm that the 7 h of food withdrawal caused an energy deficit, we analyzed plasma NEFA and glucose concentrations. Plasma NEFA concentration has been shown to serve as a reliable marker of fat mobilization in cows [61]. Increased plasma NEFA concentrations after 7 h of food withdrawal indicate a negative energy balance and were positively correlated with plasma 2-AG concentrations, whereas plasma glucose concentrations were inversely correlated with 2-AG. In support of the latter finding, a negative correlation for 2-AG with fasting glucose was also reported in humans [62]. However, an earlier study in cows showed a positive correlation between plasma AEA and plasma NEFA, but not between 2-AG and NEFA [35]. These variabilities call for further research to better understand the interaction of EC and energy metabolites.

## 5. Conclusions

The present pilot study highlights the cow as an animal model for endocannabinoid research and demonstrates that low doses of systemically administered 2-AG and AEA increase food intake in the short-term. AEA injections increased plasma AEA levels within 2.5 h after administration, indicating that i.p.-administered AEA accumulates in the circulation of cows. This finding indicates a longer half-life than only a few minutes found in mice, demonstrating the advantage of using a cow as an animal model for endocannabinoid research. However, the bioavailabilities of AEA and 2-AG are different. Despite higher plasma AEA concentrations, hypothalamic $CB_1$, AgRP, and OX-A immunoreactivity was not different between AEA and control animals, suggesting that the activation of orexigenic

neurons occurred presumably in the first hour after EC administration, as seen by the differences in food intake between groups at this time. Therefore, future studies should focus on the investigation of hypothalamic orexigenes already 1 h after i.p. EC administration. Nevertheless, downregulated NPY and AgRP mRNA expression in the ARC of AEA-treated animals indicate a counter-regulatory mechanism reducing the preceded activation of NPY/AgRP neurons. In addition, short-term food deprivation increased plasma 2-AG, while plasma AEA remained unchanged, suggesting that 2-AG is more sensitive to fasting than AEA in cows and may be more involved in the immediate response to changes in energy supply.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/nu13103587/s1, Table S1: ingredients and chemical composition of diets, Table S2: primer sequences, Table S3: *p*-values.

**Author Contributions:** I.v.A. conducted the animal experiments, provided medical care, performed statistical evaluation, and designed graphs and tables. A.K. developed the immunohistochemistry protocols. I.v.A. and B.K. wrote the manuscript. B.K. designed the animal experiment. All authors have read and agreed to the published version of the manuscript.

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# Chapter 5: Effects of endocannabinoids on feed intake, stress response and whole-body energy metabolism in dairy cows

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# Effects of endocannabinoids on feed intake, stress response and whole-body energy metabolism in dairy cows

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**Endocannabinoids, particularly anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are instrumental in regulating energy homeostasis and stress response. However, little is known about the endocannabinoid system (ECS) in ruminants, although EC could improve dairy health and productivity, at least by increasing feed intake. In this study, we report if intraperitoneal (i.p.) AEA and 2-AG administration affects feed intake, whole-body macronutrient metabolism, isolation and restraint stress, and whether diet composition modulates circulating endocannabinoid concentrations in cows. Twenty Simmental cows in late lactation were fed a grass silage and a corn silage based diet. On each diet, cows received daily i.p. injections with either AEA (5 µg/kg; *n* = 7), 2-AG (2.5 µg/kg; *n* = 6) or saline (*n* = 7) for 8 days. Endocannabinoid administration for 5 days under free-ranging (non-stressed) conditions had no effect on feed intake or energy balance, but attenuated the stress-induced suppression of feed intake when housing changed to individual tie-stalls without social or tactile interaction. Endocannabinoids increased whole-body carbohydrate oxidation, reduced fat oxidation, and affected plasma non-esterified fatty acid concentrations and fatty acid contents of total lipids. There was no effect of endocannabinoids on plasma triglyceride concentrations or hepatic lipogenesis. Plasma AEA concentrations were not affected by diet, however, plasma 2-AG concentrations tended to be lower on the corn silage based diet. In conclusion, endocannabinoids attenuate stress-induced hypophagia, increase short-term feed intake and whole-body carbohydrate oxidation and decrease whole-body fat oxidation in cows.**

The endocannabinoid system (ECS) is a complex network ubiquitously expressed throughout the body. The best studied endocannabinoids (EC) N-arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) are synthesized from membrane phospholipids, with fat depots providing an important source for plasma EC[1]. Due to their amphiphilic character, EC can modulate the activity of many membrane proteins and activate the G protein-coupled cannabinoid receptors 1 and 2 ($CB_1$ and $CB_2$) or the G protein-coupled receptor 55 (GPR55), all of them expressed at the cell surface[2,3]. Primary enzymes degrading EC are fatty acid amide hydrolase (FAAH) for the breakdown of AEA and monoglyceride lipase (MGLL) for the degradation of 2-AG. Thus, FAAH and MGLL are involved in decreasing the EC tone, contributing to the short half-life of 2-AG and AEA of only a few minutes in mice[4,5].

In rodents, a plethora of studies have shown that EC play an important role in the regulation of energy homeostasis[6] involving the control of energy intake[7–9] and energy expenditure[10]. The administration of AEA and 2-AG has been shown to stimulate feed intake of rats not only by acting at central but also peripheral sites. The injection of AEA or 2-AG into the brain[11,12] as well as into the peritoneal cavity[13] increased feed intake of rodents for a few hours. Peripherally administered EC may rapidly cross the blood–brain barrier[14] or can activate vagal afferents in the periphery, thereby triggering signaling of the gut-brain axis[15–17]. The endogenous AEA and 2-AG tone in mice can be increased by diets supplemented with EC precursors such as arachidonic acid or

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linoleic acid[18,19]. By contrast, dietary lipids with higher content in n-3 polyunsaturated fatty acids (n-3 PUFA) can reduce the EC tone and diminish $CB_1$ receptor activation in mice[20].

In addition, EC are further involved in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis[21], which in turn affects not only energy metabolism but also stress response. Restraint stress has been demonstrated to decrease the AEA, but increase the 2-AG content in the amygdala of rats[22,23]. Moreover, pharmacological enhancement of EC signaling has been shown to attenuate the response to restraint stress in rodents by reducing corticosterone release[24,25]. Furthermore, EC support anabolic metabolism by promoting fat storage and decreasing energy expenditure[26]. More specifically, activation of $CB_1$ stimulates lipogenesis in adipose cells[27] and induces the expression of the lipogenic transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) and its targets acetyl-CoA carboxylase-1 (ACC1) and fatty acid synthase (FAS) in liver[28]. However, knowledge about the involvement of the ECS in whole-body energy metabolism is mainly obtained from studies using $CB_1$ antagonists (e.g. rimonabant, which is known to exert unwanted side effects) or $CB_1$ KO mice[29,30], but there is scarcity of studies using the natural endogenous receptor agonists.

As proposed by Myers, et al.[31], activating the ECS in cows could provide the opportunity to improve dairy health and increase productivity by increasing feed intake and improving energy partitioning. Previous studies in periparturient dairy cows have shown the involvement of the ECS in adipose tissue metabolism, more specifically, an activation of the ECS in animals exhibiting high rates of lipolysis[32]. Furthermore, it has been shown that increased plasma AEA and 2-AG concentrations are directly associated with feed intake of dairy cows[33], however, a direct cause-effect relationship has not been demonstrated yet. Also, feeding a ration above energetic requirements increased the expression of the $CB_2$ receptor in bovine liver[34], but whether the diet composition affects the EC tone, or how EC affect hepatic fat metabolism in ruminants is not known.

The aim of the present study was to evaluate the effect of i.p. administered AEA and 2-AG administration on isolation and restraint stress, feed intake, milk yield, whole-body macronutrient and hepatic fat metabolism in dairy cows, and to investigate if these effects are influenced by feeding a grass silage (GS) compared to a corn silage (CS) based diet. Given the fact that the n − 6/n − 3 ratio is typically higher in CS than GS and that increased dietary n − 3 PUFA reduce the EC tone, we hypothesized that the effect of EC administration would be more pronounced on the CS diet.

## Methods

**Animals and experimental design.** Experiments were performed at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany and were in accordance with the ARRIVE guidelines (https://arriveguidelines.org), the German Animal Welfare Act and approved by the ethics committee of the State Government in Mecklenburg-Western Pomerania, Germany (Registration No. LALLF M-V 7221.3-1.1-041/18). Twenty non-pregnant Simmental cows from 1st to 10th lactation, in late lactation with an average milk yield of 15.6 ± 1.1 L per day and 263 ± 22 days in milk (DIM) were adapted to the experimental facilities for at least 20 days. The experiment was set up as a randomized block with a split plot of diet type (Supplementary Fig. S1), where animals entered the experimental trial in 5 blocks of four animals each. One block experiment lasted for 8 weeks and consisted of two 4-week feeding periods. Diets were fed in a cross over design, and the first 20 days of the second feeding period was considered the washout period. The cows were kept in a free-ranging barn and received two total mixed rations (TMR, Supplementary Table S1) based on either GS (low n − 6/n − 3 ratio) or CS (high n − 6/n − 3 ratio) with an average metabolizable energy (ME) of 10.7 ± 0.2 and 11.5 ± 0.1 MJ/kg of dry matter (DM), respectively. Troughs were filled at 05:00 a.m. and again at 05:00 p.m. Access to ad libitum intake was restricted between 05:00 a.m. and 08:00 a.m. to ensure a comparable start of feed intake. Milking took place twice daily (05:30 a.m. and 05:30 p.m.) and the milk yield was recorded. Body weight (BW) was automatically recorded when the cows passed through a scale after each milking and the metabolic BW ($mBW = BW^{0.75}$) was calculated from a weekly mean. After balancing for mBW, lactation, milk yield and DIM, animals were allocated to 3 treatment groups: 2-AG ($n = 6$), AEA ($n = 7$) and NaCl (control, $n = 7$). Treatments were administered once-daily at 08:00 a.m. during the last 8 days of each feeding period, with cows receiving the same treatment during both feeding periods. An additional 9$^{th}$ injection was given at the end of the second feeding period at 07:00 a.m. (day 9), approximately 1 h before slaughter of the NaCl-treated and approximately 2 to 3 h before slaughter of the EC-treated animals. The control group was injected 8 ml of a sterile 0.9% NaCl solution. 2-AG and AEA were administered at doses of 2.5 and 5 µg/kg BW, respectively, with a prime of twice the daily dose on day 1. The 2-AG and AEA solutions (pre-dissolved in ethanol) were purchased from Tocris (Bioscience, Bristol, UK) and diluted in 8 mL sterile 0.9% NaCl directly before administration. Doses in the low microgram/kg range were chosen because they were found to be most effective in dose response studies in rodents[9,35] and exert no unwanted cannabimimetic side effects[36,37]. Intraperitoneal injections were administered into the right paralumbar fossa (1.20 mm × 80 mm, SUPRA, Vivomed, Geislingen, Germany). The i.p. administration route was chosen to allow absorption from the peritoneal cavity into systemic circulation, as well as to activate the splanchnic ECS and vagal afferents of the gut-brain-axis[15,38]. Immediately following the injection, cows were given free access to feed. Feed intake in the barn was constantly measured with a Roughage Intake Control system (RIC, Insentec B. V., Marknesse, Netherlands) and was documented as disappearance of feed from troughs, to determine individual feed intake.

**Indirect calorimetry.** On day 6 of each treatment period, cows were transferred from the free- ranging barn into open-circuit respiration chambers and kept in individual tie-stalls[39] to induce isolation and restraint stress. Cows were fitted with an indwelling jugular vein catheter (Cavafix Certo Splittocan 338, B. Braun Melsungen AG, Melsungen, Germany) connected to a 4-m extension line to allow for blood sampling from outside of the chamber without disruption of gas exchange measurements and animal behavior. After over-night equilibration,

gas exchange measurement started on day 7 at 07:00 a.m. and lasted for 2 consecutive 24 h-periods at 15 °C. Intraperitoneal injections were administered at 08:00 a.m., and animals were fed immediately after injection and again at 05:00 p.m. for ad libitum intake. Milking in the chamber was performed at 06:00 a.m. and 04:00 p.m. Feed intake was measured as feed disappearance from trough by an electronic registration device (PAARI, Erfurt, Germany) every 6 min. The $CO_2$ and $CH_4$ concentrations were analyzed by infrared absorption (SIDOR, Sick AG, Reute, Germany) and $O_2$ concentration was analyzed paramagnetically (SIDOR) in 6-min intervals. The airflow through the chamber (approximately 10 $m^3$/h) was measured by a differential pressure type V cone flow meter (McCrometer, Hemet, CA). The mean $CO_2$ recovery rate for all 4 chambers was 99.9%.

For the following calculations, data were used as mean of both 24 h-periods. Total $CO_2$ production ($CO_{2\,total}$) is the sum of fermentative $CO_2$ ($CO_{2\,ferm}$) and metabolic $CO_2$ ($CO_{2\,metab}$). $CO_{2\,ferm}$ was estimated according to Chwalibog et al.[40] as $CO_{2\,ferm}$ (L) = 1.7 × $CH_4$ (L) in which 1.7 is constant for a variety of diet compositions[41]. Therefore, $CO_{2\,metab}$ was calculated by subtracting $CO_{2\,ferm}$ from $CO_{2\,total}$[42]. Net carbohydrate oxidation (COX) and net fat oxidation (FOX) were calculated by equations described by Frayn[43]:

$$\mathrm{COX\ (g)} = 4.55\mathrm{CO_{2\,metab}\ (L)} - 3.21\mathrm{O_2\ (L)} - 2.87\mathrm{N_u\ (g)};$$

$$\mathrm{FOX\ (g)} = 1.67\mathrm{O_2\ (L)} - 1.67\ \mathrm{CO_{2\,metab}\ (L)} - 1.92\mathrm{N_u\ (g)}.$$

The metabolic heat production (HP) was calculated according to Brouwer [44]:

$$\mathrm{HP\ (kJ)} = 16.18\ \mathrm{O_2\ (L)} + 5.02\mathrm{CO_{2\,total}\ (L)} - 2.17\mathrm{CH_4\ (L)} - 5.99\ \mathrm{N_u\ (g)}.$$

Urine N excretion ($N_u$) was not measured but estimated to 100 g/d, although real $N_u$ may vary from 75 to 150 g/d[45], thereby accepting an error in HP, COX and FOX of less than 10%.

Indirect calorimetry data was normalized to mBW and to account for individual differences among animals changes of FOX, COX, and HP were calculated relative to the start of each measurement period (07:00 a.m.). The resulting ΔFOX/mBW, ΔCOX/mBW, and ΔHP/mBW data were evaluated in hourly intervals between 07:00 a.m. and 05:00 a.m. of the following morning. In addition, daily values of FOX, COX and HP were adjusted to DMI and mBW.

**Feed and milk analyses.** Dry matter (DM) content of feed was determined weekly by drying pooled feed samples for 24 h at 60 °C followed by 4 h at 103 °C. Dry matter intake (DMI) was calculated from daily feed intake and weekly determined feed DM. During respiration chamber measurements, additional samples were taken for chemical analysis of nutrient composition. Chemical composition was analyzed by the accredited laboratory of Landwirtschaftliche Untersuchungs- und Forschungsanstalt der LMS Agrarberatung GmbH (LUFA, Rostock, Germany; Supplementary Table S1). The metabolizable energy (ME) content of the diet was calculated based on the German Society of Nutrition Physiology (GfE)[46], and the ME intake (MEI) was calculated according to MEI (MJ of ME/d) = ME (MJ/kg of DM) × DMI.

Fatty acid composition of the diets was analyzed according to Kalbe et al.[47], by using a modified method from Sukhija and Palmquist[48] for direct fatty acid methylation. The extracts were subjected to gas chromatography (GC) analysis using a CP-Sil 88 CB column (100 m × 0.25 mm, Agilent, Santa Clara, CA, United States) in a PerkinElmer gas chromatograph CLARUS 680 with a flame ionization detector (FID; PerkinElmer Instruments, Shelton, United States). The detailed GC conditions were described by Dannenberger, et al.[49] and average fatty acid composition of diets were shown in Supplementary Table S1.

Milk samples were pooled once a week from the evening and morning milking and analyzed for milk composition by infrared spectroscopy (MilkoScan; Foss GmbH, Hillerød, Denmark) at the State Inspection Association for Performance and Quality Testing Mecklenburg-Western Pomerania e.V. (LKV Güstrow, Germany). Additional samples from individual milking were taken between days 6 and 8 of the treatment period and analyzed using the same method by the Milk Testing Services North Rhine-Westphalia (LKV Krefeld, Germany). Milk composition was used to calculate the individual energy corrected milk yield (ECM) according to the GfE[50]: ECM (kg/d) = milk yield (kg/d) × ((1.05 + 0.38 × milk fat % + 0.21 × milk protein %)/3.28). Energy balance (EB) was estimated according to the GfE[50]: EB (MJ of ME/d) = MEI − (3.14 × ECM + 0.488 × mBW).

For analysis of DMI/mBW, EB, and ECM data were calculated as means during the pre-treatment period (PB, day −5 to 0), treatment under normal, non-stressed housing conditions in the barn (TB, day 1 to 5) and treatment under stressed housing conditions in the respiration chamber (TC, day 7 and 8). Additionally, percent changes from PB to TB and TC were calculated.

**Blood sampling and analyses.** To evaluate changes in fat metabolism, preprandial blood samples were collected on day 1 and day 9 at 07:00 a.m., each before EC administration in EDTA-containing tubes. Blood samples were immediately placed on ice and centrifuged at 1570 × *g* for 20 min at 4 °C, and obtained plasma was stored at −80 °C. Plasma free, non-esterified fatty acid (NEFA), triglyceride (TG) and cholesterol concentrations were analyzed spectrophotometrically with a semi-automatic analyzer (ABX Pentra 400, HORIBA Medical, Kyoto, Japan) using the following kits: NEFA-HR 91797 (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany), Triglycerides CP A11A01640 (HORIBA) and Cholesterol mono ChOD/PAP 900300 (mti-diagnostics GmbH, Idstein, Germany). The analysis of individual fatty acid concentrations in total plasma lipids was performed according to Dannenberger et al.[51]. Lipids were extracted by adding 1500 µL of plasma sample to a solution of chloroform/methanol (2:1, v/v) and internal standard (C19:0) at room temperature. Extracted lipids were methylated using sodium methylate and boron trifluoride methanol, and major fatty acids detected are listed in Supplementary Table S4. For analysis of individual changes in plasma lipid metabolites percent changes were calculated from pre-treatment to after 8 days of treatment.

For the analysis of plasma EC concentrations, plasma samples collected on day 8 prior to morning feeding and treatment were analyzed for AEA and 2-AG using the cross-validated method as described by Zoerner et al.[52]. Briefly, 18-µL of an ethanolic solution of the internal standards d5-2-AG (53.3 pg/µL) and d4-AEA (50 pg/µL) were added to the thawed plasma samples and incubated for 15 min on ice. Solvent extraction was performed by adding toluene (1 mL) and by shaking twice in a Precellys 24 Dual Homogenisator at 5000 rpm for 20 s. Phase separation was achieved by centrifugation (4655 × *g*, 4 °C, 5 min). The upper organic phase was evaporated at room temperature under nitrogen. To the residue a 40-µL aliquot of water–methanol (1:3, v/v) was added and mixed by vortexing for 10 s. Analyses were performed on a Waters ACQUITY UPLC-MS/MS system with a tandem quadruple mass spectrometer XEVO TQ MS (Waters, Milford, MA, USA). Separation of analytes was carried out on a Waters ACQUITY BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm particle size) at 60 °C.

**Liver tissue, RNA extraction and RT-qPCR.** On day 9 of EC treatment of the second feeding period, cows were stunned with a captive bolt gun and immediate exsanguinated at the institute's slaughterhouse. Liver samples were taken and immediately snap frozen in liquid nitrogen and stored at − 80 °C until further analysis. Tissue samples were ground under liquid N. RNA was extracted from 20 to 25 mg tissue powder with innuPREP RNA Mini Kit 2.0 (Analytik Jena AG, Jena, Germany) and residual DNA was removed with innuPREP DNase I Digest Kit (Analytik Jena AG). RNA concentrations were quantified spectrophotometrically on a NanoPhotometer (Implen GmbH, Munich, Germany). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), yielding RNA integrity number (RIN) factors between 7.0 and 8.7 (mean 7.7 ± 0.13). For cDNA synthesis, 750 ng total RNA was reverse transcripted (SensiFAST cDNA Synthesis Kit, Bioline, London, UK) using a Thermocycler (peqstar 96 × HPL, VWR International, Pennsylvania, USA). Transcriptional expression was quantified by real-time PCR (qPCR) using the following primers (Supplementary Table S2). qPCR was performed on a LightCycler 2.0 (Roche, Basel, Switzerland) with SensiFAST SYBR No-ROX Kit (Bioline) with 2 µL of cDNA. Each cDNA sample was analyzed in duplicate. Primer products were sequenced and the correct sequence was confirmed. The efficiency of amplification was calculated using LinRegPCR software, version 2014.4 (Academic Medical Centre, Amsterdam, Netherlands), yielding efficiency values between 1.84 and 1.92 (Supplementary Table S2). Data were quantified by qbasePlus software (Biogazelle, Gent, Belgium) and normalized to the reference genes eukaryotic translation initiation factor 3 subunit K (*EIE3K*) and peptidylprolyl isomerase A (*PPIA*).

**Statistical analyses.** Data were analyzed with mixed models (PROC MIXED) and repeated measures using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Unless otherwise stated, the random effects of all models were block and block by period interaction and a compound symmetry covariance structure was used for all data. Denominator degrees of freedom were estimated by using the Satterthwaite option in the MODEL statement and paired differences were determined using the SLICE option in PROC MIXED.

The DMI/mBW, EB, ECM, and percent changes in DMI/mBW, EB, ECM were analyzed with the fixed effects of treatment, housing, diet and all associated interactions. Housing was used in the repeated statement to account for repeated measures and the subject was defined as cow by period interaction. The ΔFOX/mBW, ΔCOX/mBW, and ΔHP/mBW were analyzed with the fixed effects of treatment, time interval, diet and all associated interactions. Time interval was used in the repeated statement and the subject was defined as cow by period interaction.

The FOX, COX and HP values adjusted to DMI and mBW as well as the percent changes in plasma lipid metabolites data were analyzed with the fixed effects of treatment, diet and their interaction. Period was used in the repeated statement and the subject was defined as cow. The mRNA abundances of hepatic genes were analyzed with the fixed effect of treatment and the random effect of block.

Plasma AEA and 2-AG concentrations were analyzed using the *t*-test procedure of SAS. Data were tested for a normal distribution with a Shapiro–Wilk test and transformed using the Johnson transformation prior to testing if necessary. Transformed data are noted in the table. Calculation of Pearson correlation between dietary fatty acids and EC concentrations was performed by using the CORR procedure of SAS. Significance was declared at $P \leq 0.05$ and tendencies were declared at $0.05 < P \leq 0.10$. Results are presented as LSM ± SE unless stated otherwise.

## Results

**Dry matter intake, energy balance and energy corrected milk yield.** DMI normalized to mBW, EB and ECM did not differ between groups pre-treatment (PB) (Supplementary Fig. S2A) and were higher on the CS than GS diet ($P < 0.05$). However, the diet had no effect on the treatment and there was no three-way interaction of diet, treatment and housing. The analysis of inidvidual changes in DMI/mBW revealed that the i.p. EC treatment under normal, non-stressed conditions in the barn (TB) for 5 days had no effect (Fig. 1a). Continuation of treatments but housing change to respiration chambers (TC) inducing isolation and restraint stress decreased DMI/mBW in all groups compared to PB ($P < 0.001$). However, the reduction in DMI/mBW from PB to TC was only 8.6 ± 3.6% with AEA and 9.6 ± 3.8% with 2-AG treatment, and was significantly less ($P < 0.01$) than in the control group (22.4 ± 3.6%, Fig. 1a). Consistent with changes in DMI/mBW, i.p. EC treatment TB had no effect on EB (Fig. 1b) and when cows were exposed to the stressful environment, EB declined ($P < 0.001$). The decline in EB was only 15.8 ± 12.6% with AEA, 28.4 ± 13.0% with 2-AG, but 60.0 ± 12.6% in the control group ($P < 0.05$, Fig. 1b). Equally, ECM was not affected by i.p. EC treatment TB and declined with change of housing to TC ($P < 0.001$; Fig. 1c). The reduction in ECM from PB to TC was 7.8 ± 3.0% with AEA, 2.5 ± 3.1% with 2-AG, but 11.7 ± 3.0% in the control group. The reduction in the control group was significantly greater than in the 2-AG group ($P < 0.05$).

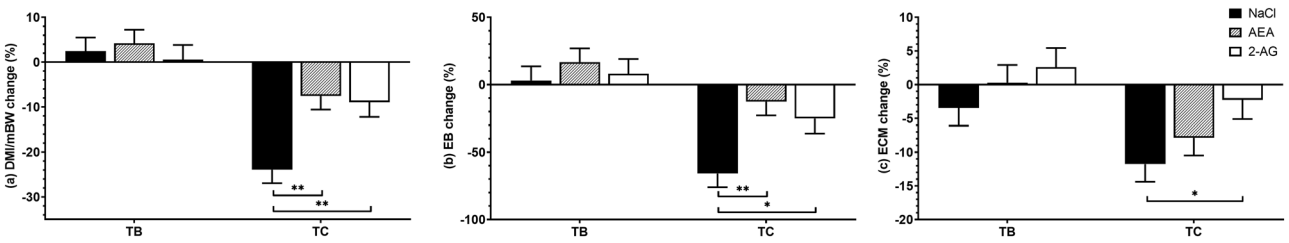


**Figure 1.** Percent changes (%) in dry matter intake (DMI) per mBW (**a**), energy balance (EB) (**b**) and energy corrected milk yield (ECM) (c) after i.p. injections with NaCl (n = 7), AEA (n = 7) or 2-AG (n = 6) under free-ranging, non-stressed conditions in the barn (TB), and under stressed conditions in the respiration chamber (TC) relative to pre-treatment. Change of housing to TC significantly decreased DMI/mBW, EB and ECM in all treatment groups ($P < 0.05$). Significant pairwise effects within-housing were detected only under stressed conditions in the respiration chamber (TC). The AEA and 2-AG treatment significantly attenuated the stess-induced decrease in DMI/mBW and EB ($P < 0.05$) and 2-AG treatment significantly attenuated the stess-induced decrease in ECM ($P < 0.05$). Graphs are presented as highest level of significant interaction, associated *P*-values can be found in Supplementary Table S3. Within-housing differences are indicated by * $P < 0.05$ and ** $P < 0.01$ (Tukey–Kramer).

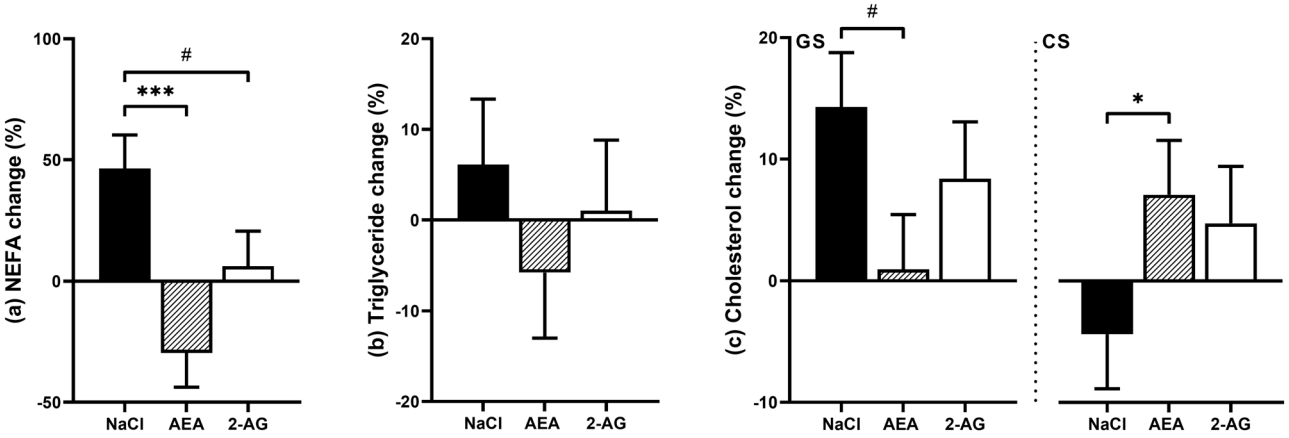


**Figure 2.** Percent changes (%) in plasma NEFA (**a**), triglyceride (TG) (**b**) and cholesterol (**c**) concentrations after i.p. injections of NaCl (n = 7), AEA (n = 7) or 2-AG (n = 6) for 8 days relative to pre-treatment. Plasma NEFA concentatrions significantly decreased after 8 days of treatment with AEA compared to the control group ($P < 0.001$) and tended to increase to a lesser extend with 2-AG treatment compared to the control group ($P = 0.05$). No changes were detected for percent changes in plasma TG. Plasma cholesterol percent changes differed in the control group between diets, as indicated by the treatment x diet interaction ($P < 0.05$). Graphs are presented as highest level of significant interaction, associated *P*-values can be found in Supplementary Table S3. Treatment differences are indicated by # < 0.1, * $P < 0.05$ and *** $P < 0.001$ (Tukey–Kramer).

**Plasma lipids.** The analysis of plasma lipid concentrations revealed some differences among animals before EC treatment, therefore, we analyzed the inidvidual changes from PB to TC. The percent changes in plasma NEFA concentrations were different between treatment groups ($P < 0.001$) (Fig. 2a). While plasma NEFA concentrations increased by 46.4 ± 13.8% in the control group and by 6.3 ± 14.5% in the 2-AG group, they decreased by 29.6 ± 14.2% in the AEA group. Resulting in a significant difference in the control group compared to the AEA group ($P < 0.001$) and a tendency to be different to the 2-AG group ($P = 0.05$). EC treatment had no effect on percent changes in plasma TG concentrations (Fig. 2b). However, TG concentration tended to be different between diets, with higher levels on the GS compared to the CS diet (10.7 vs − 9.73%; $P = 0.06$). Percent changes in plasma cholesterol concentrations differed in the control group between diets, as indicated by the treatment x diet interaction ($P < 0.05$) (Fig. 2c).

Among total lipids, the plasma palmitic acid (C16:0) content increased in the control group but declined in the AEA and 2-AG groups on both diets ($P < 0.01$; Supplementary Table S4). The oleic acid (C18:1*c*9) content also increased in the control group but remained unchanged in EC-treated animals on the GS diet and declined in EC-treated animals on the CS diet ($P < 0.01$). Palmitoleic acid (C16:1) content was affected by treatment ($P < 0.05$), with a greater decrease in the AEA and 2-AG compared to the control group. In contrast, linoleic acid (C18:2n6) was ($P < 0.01$) and stearic acid (C18:0) tended to be ($P = 0.09$) reduced in NaCl but increased or unchanged in EC-treated cows (Supplementary Table S4). Plasma arachidonic acid (C20:4n6) contents were not different between treatment groups. However, the contents of C20:4n6, C20:2n6, C20:3n6 and C22:4n6 tended to increase on the CS diet ($P < 0.1$). Percent changes in the plasma n − 6/n − 3 ratio tended to be different between treatment groups ($P = 0.05$) (Supplementary Table S4).

**Whole body energy metabolism.** Next, we examined short-term effects of EC on feed intake and whole body energy metabolism. The BW, body condition score and back fat thickness did not differ between groups (data not shown). DMI/mBW was not different between the GS and CS diet (Supplementary Table S3). Hourly DMI/mBW changed over time ($P<0.001$) and differed between treatment groups ($P<0.05$; Fig. 3a). Specifically, within the 1st h after injection, i.p. AEA and 2-AG administration resulted in a 33.8 and 49.7% higher DMI/mBW compared to the control group ($P<0.001$). Subsequently, hourly DMI/mBW was not significantly different between groups. Cumulative DMI/mBW increased over time ($P<0.001$) and tended to be differed between treatment groups ($P=0.05$; Fig. 3b). Within 8 h after i.p. injection and morning feeding, AEA and 2-AG treatment resulted in a 29.6 and 33.0% increase in cumulative DMI/mBW compared to the control group ($P<0.05$). Effects of the EC treatments were no longer observed after the afternoon feeding at 05:00 p.m. Fat oxidation (FOX) ranged from -2.0 to 14.7 g per 6 min on the GS and from -4.6 to 10.2 g per 6 min on the CS diet (data not shown). The Δ FOX/mBW varied over time ($P<0.001$; Fig. 3c) and was lower on the CS than GS diet (− 0.16 vs 0.09 g x $kg^{-1} \times kg^{-0.75}$; $P<0.05$). AEA treatment resulted in a greater decrease in Δ FOX in the first 2 and 10th h after injection and was significantly lower than in the control group ($P<0.05$). Likewise, 2-AG treatment resulted in a greater decrease in Δ FOX in the first 2 h and 10 to 12 h after injection and was significantly lower than in the control group ($P<0.05$; Fig. 3c). Carbohydrate oxidation (COX) ranged from − 4.8 to 47.1 g per 6 min on the GS and from 3.9 to 52.2 g per 6 min on the CS diet (data not shown). Δ COX/mBW varied over time ($P<0.001$) and was different between treatment groups ($P<0.01$; Fig. 3d). Specifically, i.p. EC injection resulted greater increase in Δ COX/mBW with AEA and 2-AG treatment compared to control treatment (Fig. 3d). The increase in Δ COX/mBW was significantly higher with AEA treatment 1 to 3, 5 to 14 and 19 h and with 2-AG treatment 2, 5 to 7, 10 and 13 to 14 h after injection relative to the control ($P<0.05$). Metabolic heat production (HP) normalized to mBW ranged from 380.4 to 740.7 kJ per 6 min on the GS and from 398.5 to 839.4 kJ per 6 min on the CS diet (data not shown). Δ HP/mBW was greater on the GS than CS diet (2.11 vs 0.02 $kJ \times kg^{-1} \times kg^{-0.75}$; $P<0.001$), changed over time ($P<0.001$; Fig. 3e) and was different between treatment groups ($P<0.01$; Fig. 3e). The i.p. AEA and 2-AG injections resulted in a greater increase in Δ HP/mBW. Specifically, the increase was significantly higher 3, 5 to 15 and 18 to 20 h after AEA treatment and 7 h after 2-AG treatment relative to the control ($P<0.05$).

To account for the difference in feed intake among groups during indirect calorimetry, daily FOX, COX and HP were additionally normalized to individual DMI. FOX per mBW and DMI tended to be greater on the GS than the CS diet (0.66 vs 0.33 $g \times kg^{-1} \times kg^{-0.75}$; $P=0.09$). Double normalized FOX tended to be different between treatment groups ($P=0.08$; Fig. 3f) and was 32.9% lower with AEA ($P=0.11$) and 33.5% lower 2-AG ($P=0.12$) compared to the control group. Double normalized COX tended to be higher on the CS than the GS diet (2.67 vs 2.08 $g \times kg^{-1} \times kg^{-0.75}$; $P=0.05$), but was not different between treatment groups (Fig. 3g). Double normalized metabolic HP was neither different between diets nor treatment groups (Fig. 3h).

**Transcription of Hepatic Genes.** The analysis of the mRNA abundance of hepatic genes involved in the ECS (*CNR1, FAAH, MGLL, GPR55*) and fat metabolism (*ACAA2, ACACA, CPT1A, DGAT1 and 2, HADH, PLAAT5, PPARA and SREBF1*) revealed no differences between treatment groups (Table 1).

**Plasma endocannabinoid concentrations.** We next examined if the diet composition affected the basal plasma EC concentrations. As shown in Table 2, pre-treatment plasma AEA concentration of all cows was not affected by diet composition, however, plasma 2-AG concentrations tended to be 36% lower when animals were fed the CS diet ($P<0.10$). There were no significant correlations between dietary fatty acids and plasma EC concentrations (data not shown).

## Discussion

**Effect of endocannabinoids on dry matter intake, energy balance, and isolation and restraint stress.** Many studies have shown that EC regulate feed intake in rodents[7,13,35]. To the best of our knowledge, this is the first study describing the effects of i.p. administered AEA and 2-AG on feed intake and energy metabolism in lactating dairy cows. We found that administration of EC for 5 days did not alter daily feed intake when animals were kept under free ranging (non-stressed) conditions in a barn. The lack of feed intake response may be due to a too short administration period of 5 days, a high diurnal temperature variability in the barn, social competition over preferred feeding troughs, or because short-term changes in feed intake could not be detected on the daily measurement basis (see below). With change of housing and induction of restraint stress, DMI/mBW decreased in all groups consistent with the stress-induced hypophagia in rodents and humans[53,54]. In fact, isolation of cows without social and tactile interaction has been shown to induce stress, increasing plasma cortisol and decreasing feed intake[55,56]. Furthermore, stress is exacerbated by confinement and tethering[55,57]. Another reason for the decline in feed intake is the lower energy requirement for physical activity when animals are tie-stalled. However, the decline in feed intake with housing change was significantly less with EC treatments, indicating that AEA and 2-AG administration improved habituation and attenuated the stress response. Activation of the ECS by administering the $CB_1$ agonist CP55940 or the FAAH inhibitor URB597 has shown to inhibit the restraint-induced activation of the HPA axis in mice[24]. Furthermore, CP55940 and URB597 blocked the restraint stress-induced decrease in sucrose consumption of mice[58] and ameliorated stress[59]. Although we did not measure EC when cows were kept under normal, non-stressed housing conditions, plasma 2-AG concentrations were approximately 20- to 30-fold higher during isolation and restraint stress than basal levels of cows housed in a free-ranging, non-stressed environment[33], further underscoring the involvement of EC in response to stress. Energy balance and ECM also decreased along with housing and stress level change which can be attributed to the decrease in feed intake. Furthermore, the decline in energy balance was significantly less

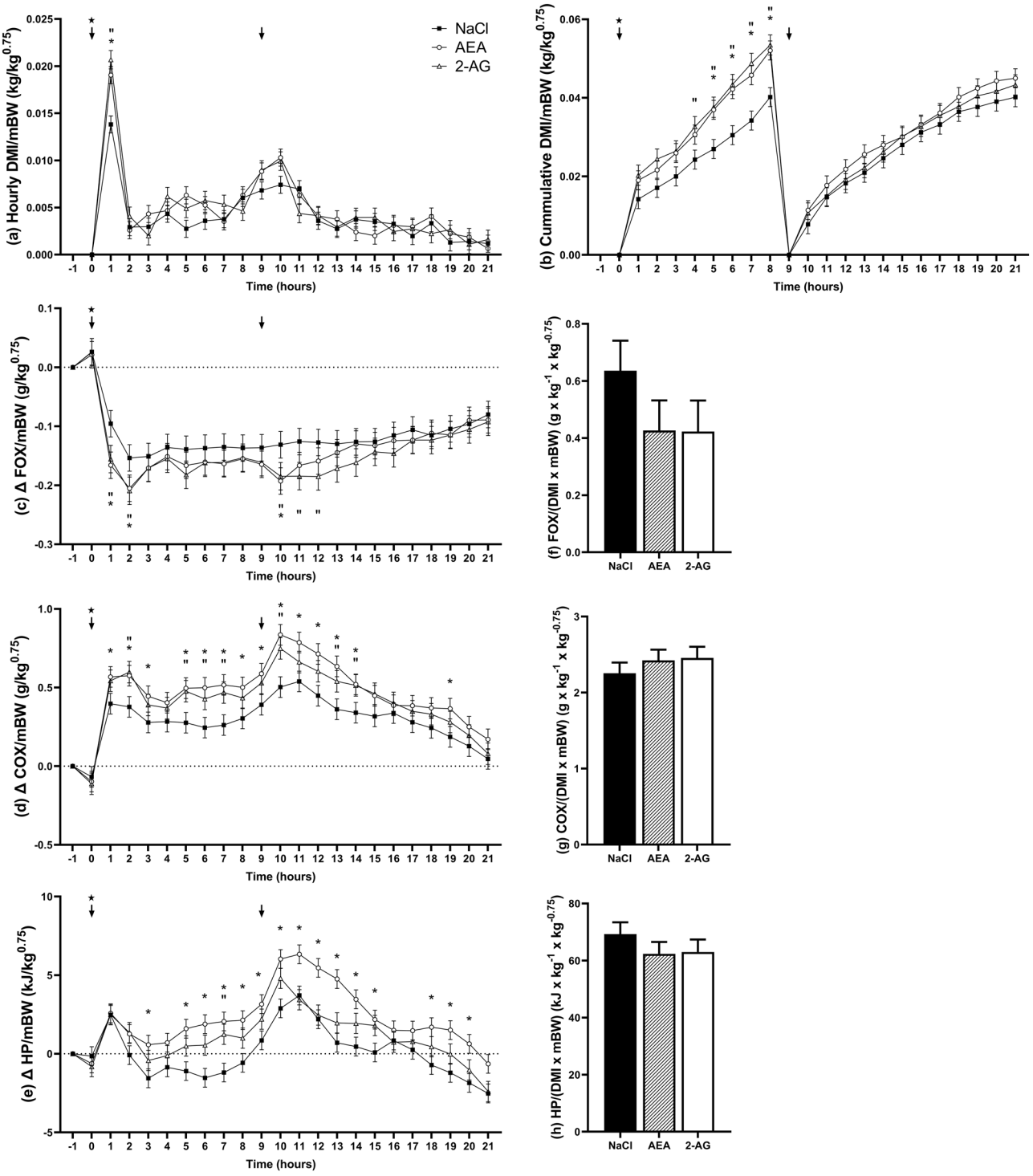


**Figure 3.** Hourly dry matter intake (DMI) per mBW (**a**), cumulative DMI/mBW (**b**), changes in fat oxidation per mBW (Δ FOX/mBW) (**c**), changes in carbohydrate oxidation per mBW (Δ COX/mBW) (**d**), changes in heat production per mBW (Δ HP/mBW) (**e**) and daily fat oxidation (FOX) (**f**), carbohydrate oxidation (COX) (**g**) and heat production (HP) (**h**), each normalized to dry matter intake (DMI) and metabolic bodyweight (mBW). Cows were intraperitoneally injected with either NaCl (n = 7), AEA (n = 7) or 2-AG (n = 6). Feed was withheld from 07:00 to 08:00 a.m. The star indicates the i.p. injection (08:00 a.m.), the arrow indicates feeding at 08:00 a.m. and 05:00 p.m. Graphs are presented as highest level of significant interaction, associated *P*-values can be found in Supplementary Table S3. Treatment differences between the control and AEA group are indicated by * ($P < 0.05$; Tukey–Kramer), and differences between the control and 2-AG group are indicated by "($P < 0.05$; Tukey–Kramer).

| Gene | NaCl | AEA | 2AG | *P*-value |
|---|---|---|---|---|
| | | | | Treatment |
| ACAA2 | 0.99 ± 0.06 | 0.99 ± 0.06 | 1.01 ± 0.06 | 0.96 |
| ACACA | 0.82 ± 0.19 | 1.25 ± 0.19 | 1.14 ± 0.21 | 0.29 |
| CNR1 | 1.38 ± 0.22 | 0.96 ± 0.22 | 1.05 ± 0.24 | 0.39 |
| CPT1A | 1.09 ± 0.10 | 0.94 ± 0.10 | 1.04 ± 0.11 | 0.43 |
| DGAT1 | 1.13 ± 0.25 | 0.92 ± 0.25 | 1.38 ± 0.27 | 0.47 |
| DGAT2 | 1.21 ± 0.25 | 1.04 ± 0.25 | 1.19 ± 0.27 | 0.87 |
| FAAH | 1.13 ± 0.08 | 0.96 ± 0.08 | 0.97 ± 0.09 | 0.23 |
| GRP55 | 1.12 ± 0.19 | 0.97 ± 0.19 | 1.20 ± 0.20 | 0.70 |
| HADH | 1.04 ± 0.06 | 0.96 ± 0.06 | 1.03 ± 0.07 | 0.61 |
| PLAAT5 | 1.38 ± 0.42 | 1.31 ± 0.42 | 1.36 ± 0.45 | 0.99 |
| MGLL | 0.97 ± 0.16 | 1.16 ± 0.16 | 1.01 ± 0.17 | 0.65 |
| PPARA | 1.09 ± 0.19 | 1.32 ± 0.19 | 0.87 ± 0.21 | 0.31 |
| SREBF1 | 1.13 ± 0.11 | 1.11 ± 0.11 | 0.86 ± 0.12 | 0.22 |

**Table 1.** Relative mRNA abundances of hepatic genes involved in endocannabinoid and fat metabolism. Liver tissue samples were obtained from cows treated with NaCl ($n = 7$), AEA ($n = 7$) or 2AG ($n = 6$) for 9 days. ACAA2, acetyl-COA acyltransferase 2; ACACA, acetyl-CoA carboxylase alpha; CNR1, cannabinoid receptor 1; CPT1A, carnitine palmitoyltransferase 1A; DGAT1/ 2, diacylglycerol O-acyltransferase 1/ 2; FAAH, fatty acid amide hydrolase; GPR55, G protein-coupled receptor 55; HADH, hydroxyacyl-CoA dehydrogenase; PLAAT5, phospholipase A and acyltransferase 5; MGLL, monoglyceride lipase; PPARA, peroxisome proliferator activated receptor alpha; SREBF1, sterol regulatory element binding transcription factor 1.

| | GS | CS | *P*-value |
|---|---|---|---|
| | | | Diet |
| AEA (nM)[1] | 0.13 ± 0.01 | 0.12 ± 0.01 | 0.53 |
| 2-AG (nM)[1] | 50.0 ± 9.46 | 31.9 ± 5.67 | < 0.10 |

**Table 2.** Plasma anandamide (AEA) and 2-arachidonoylglyerol (2-AG) concentrations (nM) of cows ($n = 20$) after ad libitum feeding of a grass silage (GS) and corn silage (CS) based diet for 27 days. Data presented as means ± SEM. [1]Data transformed using the Johnson transformation for statistical analysis and back-transformed for interpretation.

with EC treatment, thus the extent of energy balance reduction can be ameliorated by EC treatment preventing an excessive decline in energy intake.

Additionally to a stress-protective role, AEA and 2-AG administration also increased feed intake during indirect calorimetry in the short-term. The 6-min recordings showed that immediately after AEA and 2-AG injection at dosages of 5 and 2.5 µg/kg, respectively, feed intake was higher than in controls for the first hour after injection only. This short-term response may explain why we did not observe differences in daily feed intake measured in the barn. The short-term effect could be due to the observed short half-life of AEA and 2-AG of only a few minutes in heparinized mouse blood[5]. Because the half-life of 2-AG was higher in human plasma compared to rat plasma, a different half-life between various mammals can be assumed[60]. The specific half-life of AEA and 2-AG in cows is not known and needs to be determined in future studies. However, plasma AEA concentrations of the cows studied were 2.5-fold increased 2.5 h after i.p. administration of 5 µg AEA/kg BW[61]. This finding indicates that AEA accounts for the increase in feed intake despite of its relative short half-life and further suggests that the half-life of AEA in cows is presumably not as short as it is in mice. A short-term (1 to 3 h) increase in feed intake was also observed after subcutaneous administration of 0.5 to 10 mg/kg AEA to rats[7]. However, other studies performed in mice reported that AEA[35] and 2-AG[9] each administered i.p. at 0.001 mg/kg, increased cumulative feed intake over 7 or 14 days, respectively. Higher dosages tested by these authors, e.g. 0.7 and 4 mg/kg AEA[35], or 0.01 and 0.1 mg/kg 2-AG[9] had no long-term effect on cumulative feed intake in mice. Future studies are needed to determine if higher EC dosages or multiple injections per day would increase feed intake also in the long-term, but care must be taken of the risk of unwanted cannabimimetic side effects e.g. hypomobility[36,37].

**Effect of endocannabinoids on plasma lipids.** The trend for the lower DMI-normalized FOX after EC treatment was accompanied by a reduction in plasma NEFA concentrations, at least for AEA-treated animals, whereas they increased in control animals. Plasma NEFA concentrations reflect body fat mobilization and function as a quantitative marker for lipolysis in dairy cows[62,63]. Therefore, it can be assumed that animals of the control group, showing an increase in plasma NEFA concentrations, mobilized some body fat, likely because of stress-induced hyophagia in the respiration chamber. Furthermore, the reduction in plasma NEFA concentrations in AEA-treated animals and the only minimal increase in 2-AG-treated animals might indicate that EC

inhibited lipolysis in these animals despite a slight reduction in feed intake. Similarly, it has been shown that EC signaling activates lipogenic mechanisms[64], e.g. $CB_1$ activation enhanced lipogenesis in primary adipocyte cultures[27]. However, we did not measure lipolytic activity.

Total plasma fatty acids in cows can be predictive for lipid classes. While C16:0, C18:0 and C18:1c9 are the major fatty acids of the free fatty acid fraction or bound in TG, C18:2n6 is the predominant fatty acid bound in cholesterol esters and phospholipids[65]. Besides, plasma phospholipids may also contain substantial amounts of C16:0 and C18:0[65]. Our results of decreasing levels of C16:0, C16:1 and C18:1c9 after AEA and 2-AG administration for 8 days may indicate that EC facilitate the flux of free fatty acids to adipocytes for TG synthesis as described earlier[64,66]. However, plasma TG concentrations were found not different between control and EC groups. This finding is surprising as it has been reported that pharmacological stimulation of EC signaling increases plasma TG and cholesterol concentrations in mice[67]. Presumably, hypertriglyceridemia after EC treatment could not be observed in dairy cows as TG were excreted with milk, but this assumption needs further experimental approval. Plasma cholesterol concentrations increased in EC treated animals and in NaCl treated cows on the GS diet, however, they decreased in the control group on the CS diet. These results are in parallel with the stimulatory effect of EC on plasma cholesterol observed in mice[67], suggesting reduced plasma lipoprotein clearance and accumulation of plasma lipoproteins in both mice and CS-fed cows. Plasma C18:2n6 decreased in control animals, while EC administration prevented this decrease, which is likely due to the lower feed intake of animals in the control group compared to EC treatment.

**Effect of endocannabinoids on whole-body energy metabolisms.** Previous studies reporting on ECS involvement in whole-body energy metabolism mainly draw conclusions by investigating the role of $CB_1$ antagonists or $CB_1$ KO mice[29,30]. To the best of our knowledge, this is the first study describing the direct effects of i.p. administered AEA and 2-AG on whole-body energy metabolism.

Immediately after i.p. injections and morning feeding, whole body fat oxidation decreased while oxidation of carbohydrates increased in all groups, which is due to the high carbohydrate content in feed. An inverse relationship between COX and FOX, and between FOX and feed intake but a positive relationship between COX and feed intake has been reported earlier for dairy cows[42]. Accordingly, the higher feed intake after AEA and 2-AG treatment was accompanied by a higher COX, not only after the morning feeding but also in the afternoon. The longer-lasting effect in higher COX can be explained by the fact that more feed ingested with the morning feeding requires longer fermentation and digestion time which in turn delay metabolic $CO_2$ production from dietary carbohydrates in post-absorptive metabolic processes. In parallel to the short-term response in feed intake after EC injection, the reduction in FOX was most pronounced in the first two hours after injection. Both FOX and COX contribute to total metabolic HP since the portion of heat produced from COX is higher than from FOX, and since COX and feed intake were higher in EC- compared to NaCl-treated cows, the resulting metabolic HP was higher in AEA and 2-AG treated animals.

To account for the differences in feed and energy intake between treatment groups and to examine whether changes were limited to a feed intake-related effect, daily FOX, COX and metabolic HP were additionally normalized to DMI. The analysis revealed that double normalized FOX tended to be lower in the AEA and 2-AG compared to the control group, suggesting that effects extend beyond differences in feed intake and that AEA and 2-AG suppress whole-body fat catabolism and/or increase lipogenesis. These findings support earlier indirect calorimetry studies reporting that the $CB_1$ antagonist rimonabant increased energy expenditure in rats by increasing fat oxidation[29,30]. From these studies and since endocannabinoids were reported to favor anabolic processes[26,68], we expected double normalized metabolic HP to be reduced in the AEA and 2-AG compared to the control group, but the EC effect could not be tested significantly lower. Apparently, the numeric increase in DMI-normalized COX overrides the tending FOX decrease induced by EC.

**Effect of endocannabinoids on hepatic gene expression.** The expression of hepatic genes involved in the ECS and fat metabolism were not different between treatment groups. Our results are in contrast to studies performed in mice, where $CB_1$ activation increased de novo fatty acid synthesis and increased hepatic gene expression of genes involved in lipogenesis, such as SREBP-1, ACC1 and FAS[28]. In contrast to monogastric species, the bovine liver is only marginally capable to perform de novo fat synthesis because of limited ACC abundance, whereas adipose tissue accounts for over 92% of whole-body fatty acid synthesis[69]. The lack of expression differences between treatment groups may also be due to the fact that i.p. administered EC had no direct effect on liver, or because of the high inter-individual variation in gene expression.

**Effect of diets on plasma endocannabinoid concentrations.** Recent studies in mice have shown that feeding an elevated dietary linoleic acid (C18:2n6) content increases the AEA and 2-AG concentrations in brain and liver tissue[18,70]. The linoleic acid content of the diets tested in the present study was 12.9% higher in the CS than GS diet. Although we found numerically higher plasma C18:2n6 content in animals fed the CS diet (data not shown), plasma AEA concentrations were comparable between, and 2-AG plasma concentrations were even lower with CS feeding. Presumably, the 30% difference in the dietary linoleic acid content was not large enough to induce differences in plasma EC concentrations. This conclusion is supported by earlier studies reporting approximately twofold higher tissue EC levels with feeding an eightfold higher linoleic acid content[18,70]. However, the tending lower plasma 2-AG concentrations with CS feeding might be explained by the higher content n-3 PUFA, known to reduce the EC tone[20], yet it remains questionable why this difference is not observed for AEA. Besides the different amounts of some constitutes in the rations, a major reason might be the lower energy intake of the animals on the GS diet, as the reduction of energy intake has been shown to stimulate 2-AG biosynthesis[71].

## Conclusion

The present study demonstrates that i.p. administration of AEA and 2-AG reduces the stress-induced suppression of feed intake and stimulates feed intake in the short-term. As a consequence of the latter, both EC increase whole-body carbohydrate oxidation and metabolic heat production. EC administration reduces whole-body fat oxidation exceeding differences in feed intake, suggesting that AEA and 2-AG suppress whole-body fat catabolism and/or support lipogenesis. However, once daily EC administration do not affect plasma TG concentrations, hepatic lipogenesis and hepatic fatty acid oxidation in dairy cows. Future studies should also determine if higher EC dosages, multiple injections per day or continuous EC infusion would alter feed intake and metabolism in the long-term. Finally, feeding a corn silage based diet with a 30% higher linoleic acid content than grass silage did not increase plasma EC concentrations, thus more research is required to elucidate how diet formulation can modulate the EC tone in ruminants.

## Data availability

All data generated and analyzed are available from the corresponding author on request.

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## Author contributions

The authors' contributions were as follows: I.v.A. conducted the animal experiment, provided medical care, performed statistical evaluation, and designed graphs and tables. A.T. developed the statistical models. R.W. and D.D. performed lipid extraction and GC-FID analysis. I.v.A. and B.K. wrote the manuscript. B.K. designed and conducted the animal experiment. All authors approved the final manuscript as submitted.

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# Chapter 6: Discussion

The endocannabinoid system (ECS) is ubiquitously expressed throughout the body and has been identified as an integral part in the regulation of energy homeostasis and the physiological adaptation to major metabolic changes (Ruiz De Azua and Lutz 2019). Among others, activation of the ECS promotes feed intake via central and peripheral mechanisms (Matias et al. 2006a) and energy partitioning by adipocyte proliferation, glucose uptake, lipid accumulation, and suppressing lipolysis (Vettor and Pagano 2009). As outlined in the literature review (Chapter 2.3), research regarding the ECS in dairy cows is still limited. In a recent review, Myers et al. (2021) outlined current research developments of ECS-involvement in periparturient cows, and highlighted the potential therapeutic implications of modulating ECS activity to one day improve dairy health and productivity. In the present thesis, the main focus was to investigate the involvement of the ECS in maintaining energy homeostasis of late-lactating cows and evaluate the effects of intraperitoneally (i.p.) administered AEA and 2-AG. Although the transition period, which poses a major challenge to overall health and productivity of dairy cows, is of interest for potential therapeutic intervention, the animal study of this thesis was conducted in late-lactating cows. Since this line of studies was the first to test the effects of i.p. administered endocannabinoids, we wanted to exclude known effects of reduced hormonal sensitivities or possible metabolic disorders associated with parturition and the transition from the non-lactating to the lactating state (e.g., milk fever and ketosis) (Vergara et al. 2014). Furthermore, we strictly used the endogenous cannabinoid receptor agonists AEA and 2-AG instead of uptake inhibitors or synthetic receptor agonists to (i) study the effect of true endocannabinoids and (ii) to allow cows to continue to be used as food-producing animals.

### Changes in endocannabinoid tone in response to feeding, stress, diet composition, and i.p. endocannabinoid administrations in dairy cows

In non-ruminants, the endocannabinoid tone has been described as highly dynamic (Hillard 2018) and plasma concentrations are known to be modulated by a number of physiological and non-physiological factors (Chapter 2.1.3). Until now, only few studies had investigated plasma endocannabinoid concentrations in cows. Artegoitia et al. (2016) and Artegoitia et al. (2017) were the first to describe AEA and 2-AG plasma concentrations in cows and showed a positive correlation between plasma AEA and feed efficiency; however, these studies were performed in beef cattle. Subsequently, Kuhla et al. (2019) described plasma endocannabinoid levels in dairy cows during the transition from late pregnancy to early lactation. The latter study demonstrated an increase in postpartum plasma endocannabinoid concentrations, which were associated with elevated energy intake and metabolic rate in early lactation. However, a direct

cause-effect relationship could not be derived from this study. Therefore, to further understand the dynamics of the endocannabinoid tone in dairy cows, different physiological and non-physiological influencing factors were tested in a set of studies within this thesis. Further considering that endocannabinoids are highly lipophilic and can equilibrate from tissues into the circulation, Hillard (2018) suggested that plasma endocannabinoid concentrations can serve as indirect markers of the tissue endocannabinoid tone. Based on this presumption and to minimalize invasive interventions in the studied dairy cows, AEA and 2-AG concentrations were analyzed in plasma only.

Physiologically, the endocannabinoid tone reacts quickly to changes in the metabolic status, depending on the level of energy supply (Chapter 2.1.3). Accordingly, in non-ruminants, an energy deficit induced by fasting increased the 2-AG concentration in the hypothalamus (Kirkham et al. 2002), and of both 2-AG and AEA in the limbic forebrain and small intestine (Kirkham et al. 2002; Dipatrizio et al. 2015), whereas refeeding has been shown to immediately reverse this effect. To investigate whether the metabolic status (level of energy supply) is also reflected in the endocannabinoid tone of ruminants that have a continuous nutrient supply from the rumen, cows were subjected to short-term feed deprivation (7 h) and plasma samples were analyzed for AEA and 2-AG concentrations (Chapter 4). Findings in dairy cows were consistent with those in non-ruminants, at least for 2-AG, since plasma 2-AG levels in cows also increased after feed deprivation and returned to baseline levels after refeeding (Chapter 4). Plasma AEA concentrations in cows remained unchanged (Chapter 4). However, this finding is not fully unexpected given that not all studies in non-ruminants were able to provide consistent results for AEA after feed deprivation (Gomez et al. 2002; Kirkham et al. 2002). In fact, variabilities in response to energy deficits could be related to time-dependent variations in endocannabinoid levels (Hanus et al. 2003). On the other hand, it is also plausible that feed deprivation of only 7 h in dairy cows did not achieve full fasting conditions and therefore did not trigger the upregulation of AEA.

In addition, the endocannabinoid tone is also affected by stress. More specifically, a stress-induced modulation of the endocannabinoid tone has been described in non-ruminants (Chapters 2.1.3 and 2.2.2), leading to a reduction in AEA but an increase in 2-AG levels in the brain (Patel et al. 2005; Rademacher et al. 2008; Hill et al. 2010b). Consistently, plasma 2-AG concentrations of stressed dairy cows were approximately 20- to 30-fold higher (Chapter 4) compared to basal levels of cows kept in a free-ranging, non-stressed environment (Kuhla et al. 2019) or even compared to feed deprived, free-ranging cows (Chapter 4). These highly elevated plasma 2-AG levels reflect the previously described progressive increase with prolonged stress in non-ruminants (Morena et al. 2016) (Chapter 2.2.2) and indicate a systemic upregulation of 2-AG. However, plasma AEA levels were comparable between non-stressed and stressed dairy cows. Taken together, the physiological changes in endocannabinoid levels

in the plasma of dairy cows are mostly in line with those in the tissue of non-ruminates, further strengthening the aforementioned hypothesis of Hillard (2018). However, in dairy cows, 2-AG appears to be more involved in the systemic metabolic response to feed deprivation than AEA.

Non-physiological influencing factors of the endocannabinoid tone include, among others, variations in dietary constituents, e.g., changes in the n6/n3 ratio (Chapter 2.1.3), given that the n6 fatty acid linoleic acid can serve as an indirect precursor for endocannabinoid biosynthesis (Kim et al. 2013). In fact, in non-ruminants eightfold elevated dietary linoleic acid contents have been shown to increase endocannabinoid levels in brain, bowel and liver (Alvheim et al. 2012; Alvheim et al. 2014; Ghosh et al. 2019). However, if this is also true for dairy cows has not been tested so far. In our study, feeding a high n6/n3 diet (based on corn silage) versus a low n6/n3 diet (based on grass silage) did not increase plasma endocannabinoid concentrations, although plasma linoleic acid concentrations were in fact elevated in cows fed the high n6/n3 diet (Chapter 5). Either the 30% difference in n6/n3 ratio was not sufficient to induce systemic endocannabinoid biosynthesis, or changes were confined to single tissues. Unfortunately, we were not able to obtain adequate samples to examine the respective tissue endocannabinoid concentrations. Therefore, to further elucidate the effect of changes in the n6/n3 ratio, a more in-depth study examining tissue samples would be necessary.

To support the approach of pharmacological enhancement of the endocannabinoid tone in dairy cows, we further analyzed plasma AEA and 2-AG levels 1 h before, 2.5 and 5.5 h after i.p. administration (Chapter 4). Compared with pre-injection levels, plasma AEA concentrations were elevated 2.5 h after AEA injection and subsequently declined back to baseline levels, whereas plasma AEA levels in the control (NaCl) and 2-AG group remained unchanged (Chapter 4). 2-AG concentrations remained constant in the collected samples regardless of whether AEA, 2-AG or NaCl was injected (Chapter 4). Most likely, we missed the time of peak accumulation in the circulation, since we analyzed only 2.5 h post-injection. Nevertheless, our results may indicate that administered AEA is more stable in dairy cows than in rodents. The availability and duration of action of AEA in rodents has indeed been shown to be limited (Smith et al. 1994) and when incubated with mouse blood, synthetic 2-AG has been shown to degrade rapidly (< 2 minutes), whereas AEA appeared to be marginally more stable (> 5 minutes) (Járai et al. 2000). However, a later study by Willoughby et al. (1997) could show a biphasic pattern in radioactivity enrichment in plasma and various tissues after i.v. administration of tracer-labeled AEA in mice. Taken together with the intracellular accumulation of AEA in adiposomes as suggested by Oddi et al. (2008), administered AEA might have been transiently stored in the adipose tissue of cows and possibly re-entered the circulation with a time delay. However, a pharmacokinetic study with higher frequency sampling would be

necessary to define tissue distribution in cows and clarify the differing bioavailabilities of AEA and 2-AG.

**Endocannabinoid-mediated effects on feed intake and hypothalamic orexigenic signaling in dairy cows**

The ECS plays a pivotal role within the complex control and regulation of feed intake (Chapter 2.2.1). In general, pharmacological activation of the ECS with either endocannabinoids or synthetic analogues has been repeatedly shown to increase feed intake in non-ruminants (see below). Both peripherally and centrally administered endocannabinoids induced hyperphagia and increased energy intake (see below). To this point, however, the effect of endocannabinoid administration on feed intake has not yet been studied in dairy cows. Although Kuhla et al. (2019) showed a link between plasma AEA and 2-AG concentrations and feed intake, the direct cause-effect relationship remained to be established and was therefore a further focus of this thesis. Among administration routes, studies in non-ruminants have shown that the commonly used intraperitoneal (i.p.) injection (Hao et al. 2000; Gomez et al. 2002; Avraham et al. 2017), as well as subcutaneous (Williams and Kirkham 1999), and intracerebral (Jamshidi and Taylor 2001; Kirkham et al. 2002; Chapman et al. 2012) administrations of endocannabinoids led to a robust increase in feed intake. However, in our line of investigation, we have chosen the i.p. administration route not only to allow absorption from the peritoneal cavity into systemic circulation, but also to directly activate the splanchnic ECS and vagal afferent neurons of the gut-brain-axis (Burdyga et al. 2010; Dipatrizio 2021). Furthermore, in this way, cows were treated in the most practical and least invasive manner when administered an i.p. injection. The very invasive and cost-intensive intracerebral, or more specifically intracerebroventricular, injection was also recently tested for AEA in cows (Kuhla and Van Ackern 2022), with similar outcomes as the i.p. injection in terms of feed intake. However, whether the i.v. or i.m. injection routes also serve as reliable ways to activate the ECS in dairy cows needs to be evaluated in the future. Dosages chosen for AEA and 2-AG injections were in the low microgram/kg range because they were found to be most effective in preliminary investigations in dairy cows and dose-response studies in non-ruminants (Hao et al. 2000; Avraham et al. 2017) without inducing unwanted cannabimimetic side effects, e.g., hypomobility (Sulcova et al. 1998; Long et al. 2009). In contrast to some studies in non-ruminants, where using comparably low doses of AEA or 2-AG increased cumulative feed intake over 7 or 14 days (Hao et al. 2000; Avraham et al. 2017), neither AEA nor 2-AG administration had a long-term effect on feed intake in dairy cows (Chapter 5). Only the in-depth study of short-term feed intake using 6-minute interval recordings obtained in respiration chambers and the analysis of hourly intervals revealed an

effect of the endocannabinoid treatment in cows (Chapter 4, Chapter 5). The i.p. 2-AG and AEA administration resulted in higher feed intake compared to the control group within the first hour after treatment but did not differ between treatment groups in the following hours. This brief effect may explain why we were not able to detect treatment differences for daily and long-term measurements. Another reason why endocannabinoid treatment only increased feed intake during respiration chamber measurements could be that observed effects only became apparent with the upregulated endocannabinoid tone upon the onset of isolation and restraint stress (see plasma levels above and discussion below). Interestingly in dairy cows, plasma AEA concentrations were still elevated 2.5 h after AEA administration, but effects on feed intake only lasted one hour. This discrepancy calls for further investigation of the bioavailability of administered endocannabinoids in dairy cows. Moreover, future studies are needed to determine if higher dosages or multiple injections per day would increase feed intake in the long-term, consequently potentially improving dairy health and productivity by promoting feed intake according to Myers et al. (2021).

The ECS has previously been shown to play an integral part in the central control of feed intake (Koch 2017). The endocannabinoids AEA and 2-AG function as lipophilic signaling molecules and are able to activate hypothalamic neurons and modulate the release of various neurotransmitters (Di Marzo et al. 1998). Endocannabinoids, being highly lipophilic molecules with a low molecular weight, are able to be rapidly absorbed from the peritoneal cavity into systemic circulation (Al Shoyaib et al. 2019). Furthermore, studies in non-ruminants have shown that peripherally administered endocannabinoids rapidly cross the blood-brain barrier (BBB) (Fride and Mechoulam 1993), and that radiolabeled AEA was detectable in the brain of mice as early as 1 min after i.v. injection (Willoughby et al. 1997). Moreover, peripherally administered endocannabinoids can trigger signaling of the gut-brain axis by binding to $CB_1$ receptors expressed by vagal afferent neurons (Storr and Sharkey 2007; Burdyga et al. 2010; Dipatrizio 2016), thereby, activating hypothalamic signaling and increasing feed intake. In non-ruminants, endocannabinoids can exert neuromodulatory effects by activating major orexigenic neurons located either in the arcuate nucleus (ARC), e.g., neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons, or in the lateral hypothalamus, e.g., orexin-A (OX-A) neurons (Chapter 2.2.1). Unfortunately, the investigation into the immunoreactivity or c-Fos activation of selected neuropeptides ($CB_1$, AgRP, OX-A) revealed no neuromodulatory effect of either AEA or 2-AG 2 to 3 hours after injection (Chapter 4). Following the hypothesis that i.p. administered endocannabinoids in dairy cows are also rapidly absorbed form the peritoneal cavity into the systemic circulation and rapidly cross the BBB (see above), we expected to find activation of hypothalamic neuropeptides after enhancing the systemic endocannabinoid tone. However, failure to detect increased activation of hypothalamic neurons might have been due to increased stress levels during respiration chamber measurements prior to slaughter and the

transport of the animals to the slaughterhouse, which may have suppressed endocannabinoid-induced neuron activation. In fact, Morena et al. (2016) established that exposure to stress causes downregulation of $CB_1$ receptors in the brain of non-ruminants. Another reason might have been the 2 to 3 hour time delay between i.p. injection and tissue collection. Speculatively, activation of orexigenic neurons might have occurred in the first hour after endocannabinoid administration, in line with increased feed intake but not in parallel with elevated plasma AEA concentrations. Therefore, future studies should focus on the investigation of hypothalamic orexigenes within 1 h after i.p. endocannabinoid administration and feeding with minimal exposure to stress. Moreover, expression of genes related to the ECS in the ARC and PVN of dairy cows was not different between treatment groups. However, NPY and AgRP RNA abundances were downregulated in the ARC of AEA-treated animals. This finding might indicate a possible counter-regulatory mechanism that reduced the preceding activation of NPY/AgRP neurons. This hypothesis is further supported by the highly increased AEA plasma concentrations around the same time of slaughter and sample collection 2 to 3 hours after i.p. administration. Another important aspect, which we have not investigated, is that endocannabinoids also interact with anorexic circuits (Chapter 2.2.1). For example, $CB_1$ receptor activation mediates downregulation of POMC synthesis and α-MSH release in the ARC of non-ruminants (Morello et al. 2016). Also, not part of our study was the investigation of hedonic aspects of feed intake regulation, the gut-brain axis, or the interaction of endocannabinoids with peripheral hormones or adiposity signals, e.g., CCK, leptin, or ghrelin (Chapter 2.2.1). However, for further understanding of the ECS in dairy cows these aspects should be considered in future studies.

**Endocannabinoid-mediated amelioration of stress-related suppression of feed intake in dairy cows**

Within the body's response to stress the ECS is integrated in several regulatory processes (Chapter 2.2.2). However, remaining within the focus of this thesis, investigations concerning endocannabinoid involvement in the stress response were limited to the exploration of feed intake and adaptation to stress. In non-ruminants, activation of the stress response, involving the hypothalamic-pituitary-adrenal (HPA) axis, can negatively affect appetite and feeding behavior (Ulrich-Lai et al. 2015). The ECS acts to constrain the magnitude of the stress response and promote the return to a non-stressed state (Morena et al. 2016). Recent studies in non-ruminant have demonstrated that pharmacological activation of the ECS attenuates stress-induced alterations in feed intake (Sticht et al. 2019) and attenuates corticosterone release (Patel et al. 2004; Hill et al. 2009). Corresponding to non-ruminants, stress exposure has also been shown to suppress feed intake in dairy cows (Herskin et al. 2007; Llonch et al.

2018). However, whether cows may also benefit from pharmacological activation of the ECS in response to stress has not yet been investigated. In this thesis, we compared the feed intake of the same cows in a non-stressed versus stressed environment with either AEA, 2-AG, or control treatment (Chapter 5). Stress was induced by isolation of cows without social and tactile interaction and tethering in individual tie-stalls in respiration chambers. Although we did not measure stress parameters, e.g., cortisol, it has been previously established that confinement of cows in respiration chambers (Llonch et al. 2018), as well as social isolation and restraint (Herskin et al. 2007; Szenci et al. 2011), leads to a robust stress response with a reduction in feed intake and an increase in plasma cortisol levels. In our study, administered endocannabinoids increased feed intake only in the short term (see above). However, when considering the reduction of feed intake with the onset of stress it became apparent that the treatment of cows with AEA or 2-AG attenuated stress-induced hypophagia and might have improved habituation to the stress (Chapter 5). Interestingly, based on the bidirectional regulation of AEA and 2-AG after stress exposure (Chapter 2.2.2), it has been suggested that mainly 2-AG is involved in the adaptation to stress (Morena et al. 2016) by mediating a rapid negative feedback inhibition of the HPA axis, whereas AEA provides more of a steady-state inhibition of HPA axis activity (Hill et al. 2010c). However, we did not observe differences in the attenuation of stress-induced hypophagia between 2-AG and AEA treatments. Injecting differing doses of AEA and 2-AG of 5 µg/kg and 2.5 µg/kg could have had an influence on the stress protective role. In the future, it would be interesting to test different dosages to study the possibility of managing stress in dairy cows and improving productivity.

**Endocannabinoid-mediated changes in whole-body metabolism in dairy cows**

The ECS is involved in many aspects of metabolic control, but the extent that this involvement is reflected when the whole organism is considered is not well established (Chapter 2.2.3). Studies that have addressed this issue and performed gas exchange measurements in respiration chambers are scarce but are indispensable for indirect calorimetry calculations and subsequent predictions of whole-body macronutrient metabolism. As stated above (Chapter 2.2.3), the existing studies in non-ruminants did not use endogenous receptor agonists, but mainly draw conclusions based on the effects of the $CB_1$ receptor antagonist rimonabant (Herling et al. 2008; Kunz et al. 2008) or the $CB_1$ receptor knock out (Cardinal et al. 2012). Therefore, it is mostly speculation to say that ECS activation decreases whole-body energy expenditure or fat oxidation (FOX) (Kunos et al. 2008; Cavuoto and Wittert 2009). To the best of our knowledge, regardless of the species, the study conducted as part of this thesis was the first to investigate the direct effects of i.p. administered AEA and 2-AG on whole-body energy metabolism (Chapter 5). Based on the obtained results, we were able to gain new insights that

are at least partially consistent with the aforementioned assumptions. Fundamentally, it is well established that COX is positively, and FOX is inversely correlated with feed intake in dairy cows (Derno et al. 2013). Consequently, in accordance with the endocannabinoid-mediated short-term increase in feed intake in cows (see above), AEA and 2-AG treatment led to a higher increase in COX compared to control treatment (Chapter 5). Interestingly, the effect on COX seemed to persist over several hours, which most likely reflected that the additional amount of feed ingested required a longer fermentation and digestion time, which in turn delayed and amplified metabolic $CO_2$ production from dietary carbohydrates in post-absorptive metabolic processes. In parallel, the endocannabinoid-mediated short-term increase in feed intake was accompanied by a greater reduction in FOX with AEA and 2-AG treatment, especially within the first two hours after treatment. Moreover, the metabolic heat production (HP) also increased more in cows treated with endocannabinoids, which is plausible because both COX and FOX contribute to metabolic HP and the portion of heat produced from COX is higher than from FOX. However, to account for the differences in feed intake between treatment groups and to examine whether observed changes were limited to a feed intake-related effect only, values of COX, FOX, and HP were additionally normalized to feed intake (Chapter 5). In fact, when analyzed independently of feed intake, COX was not different between AEA and 2-AG groups, whereas FOX remained lower with AEA and 2-AG treatment relative to the control (Chapter 5). The latter finding indicates that effects of AEA and 2-AG treatment indeed suppressed whole-body fat catabolism and/or increased lipogenesis, further underlining the anabolic nature of the ECS (Kunos et al. 2008; Richard et al. 2009). This further elucidates the question raised below as to whether lowered plasma non-esterified fatty acid (**NEFA**) levels associated with endocannabinoid treatment are related to feed intake or actually due to the inhibition of body fat mobilization (see below). However, these findings need to be further validated by more in-depth analysis of the underlying molecular mechanisms. Contrary to expectations, HP could not be tested significantly lower with endocannabinoid treatment when analyzed independently of the feed intake. Additionally, effects were possibly time limited and were not captured by the analysis of daily data. Therefore, further studies should investigate different doses of AEA and 2-AG administration to determine if there is a more effective dose with longer acting effects.

**Endocannabinoid-mediated alterations in lipid metabolism in dairy cows**

The involvement of the ECS in lipid metabolism is not fully understood. However, current research in non-ruminants suggests activation of the ECS in cells associated with lipid metabolism stimulates energy accumulation (Chapter 2.2.4). Specifically, activation of the ECS has been shown to increase adipocyte proliferation and differentiation (Bellocchio et al. 2008b), stimulate lipogenesis (Cota et al. 2003; Osei-Hyiaman et al. 2005), and limit fat mobilization

(Buch et al. 2021). In dairy cows, a number of studies have investigated the ECS in the adipose tissue, focusing on the periparturient period, which represents a major metabolic challenge. As a pioneer, Zachut et al. (2018) described the presence of the ECS in the adipose tissue of dairy cows, which could be linked to the metabolic adaptation during the onset of lactation. In particular, the ECS appears to exert a protective role to counter the increased lipolysis in high weight loss cows by upregulating expression of CB1 and levels of AEA and 2-AG in adipose tissue (Zachut et al. 2018). A study by Dirandeh et al. (2020) supports this assertion by linking the degree of body condition score loss to the activation of the ECS in the subcutaneous adipose tissue in cows at the onset of lactation. Consequently, Myers et al. (2021) argued that targeting the lipid metabolism by activating endocannabinoid signaling in postpartum cows could potentially reduce body fat mobilization and could therefore reduce hepatic accumulation of TAG and synthesis of ketones improving overall health. However, no study has investigated whether direct pharmacological augmentation of the endocannabinoid tone affects lipid metabolism in cows. Preliminary investigation of changes in fat metabolism after AEA and 2-AG administration in late-lactating cows investigated in the present study revealed individual changes in plasma lipids (Chapter 5). Injected endocannabinoids attenuated the increase in plasma NEFA associated with stress-induced hypophagia (Chapter 5). In dairy cows, plasma NEFA concentrations reflect body fat mobilization and function as a quantitative marker for lipolysis (Bobe et al. 2004; Petit et al. 2007). Therefore, it is plausible that endocannabinoid treatment might have inhibited body fat mobilization. The question arose, if this effect was due to the endocannabinoid-mediated attenuation of stress-induced reduction in feed intake or due to an underlying inhibition of lipolysis exceeding the differences in energy intake. Regrettably, we did not measure lipolytic activity, but measurements of whole-body fat oxidation in respiration chambers provided some insight (see above). Among total plasma lipids, palmitic acid (C16:0), palmitoleic acid (C16:1), and oleic acid (C18:1c9) concentrations in cows decreased after 8 days of AEA and 2-AG treatment (Chapter 5). This finding might indicate an endocannabinoid-mediated flux of free fatty acids to adipocytes for triglyceride synthesis similar to earlier described findings in non-ruminates (Bellocchio et al. 2008b; Vettor and Pagano 2009). However, plasma triglyceride concentrations did not reflect this assumption (Chapter 5), and we were not able to collect samples from adipose tissue for verification. Additionally, in the liver of non-ruminants, activation of the ECS has been shown to increase de novo fatty acid synthesis and increased hepatic gene expression of genes involved in lipogenesis, such as SREBP-1, ACC1 and FAS (Osei-Hyiaman et al. 2005). However, analysis of hepatic gene expression in dairy cows revealed no effect of administered endocannabinoids on genes involved in the ECS or hepatic lipogenesis and fatty acid oxidation (Chapter 5). Consequently, investigations of the effects of administered endocannabinoids on lipid metabolism remain superficial and further studies focusing on adipose tissue are required. A

follow up study in periparturient dairy cows is currently examining repeated biopsies from subcutaneous adipose and liver tissue and can hopefully provide more insight.

**Conclusions**

In dairy cows, the ECS is a relatively new field of study with limited insights so far. The studies included in this thesis examined the dynamics of the endocannabinoid tone in dairy cows and tested the direct effects of i.p. administered endocannabinoids on feed intake, hypothalamic orexigenic signaling, stress response, whole-body and lipid metabolism.

In line with research in non-ruminants, our studies show that metabolic changes in dairy cows, e.g., feed deprivation and stress, dynamically influence the endocannabinoid tone by increasing plasma 2-AG concentrations. Yet, in dairy cows, 2-AG appears to be more involved in the systemic metabolic response to feed deprivation and stress exposure than AEA, as plasma AEA levels remained unchanged during these challenges. However, we could only cover a limited period of time and presumably more frequent tissue sampling is needed for a full profile of the physiological dynamics of certain factors in the endocannabinoid tone in dairy cows. Contrary to non-ruminant species, plasma endocannabinoid levels in cows did not increase by altering the n6/n3 ratio in the diet. Furthermore, we showed the systemic distribution of i.p. administered AEA in dairy cows in plasma samples 2.5 h after injection, but not for 2-AG. Bioavailabilities of injected AEA and 2-AG seem to differ, and findings indicate a certain accumulation of AEA in the circulation or possibly the adipose tissue of cows. Additional research is required to determine the pharmacokinetic properties of administered endocannabinoids in cows.

Despite the apparent difference in the duration of plasma availability of injected AEA and 2-AG in dairy cows, both treatments led to a short-term increase in feed intake only within the first hour after injection and attenuated stress-induced hypophagia but had no long-term effect on feed intake. The investigation of hypothalamic orexigenic signaling in dairy cows revealed no neuromodulatory effect of either treatment on immunoreactivity or c-Fos activation of selected neuropeptides ($CB_1$, AgRP, OX-A) between 2 and 3 hours after injection. However, the downregulation of NPY and AgRP mRNA abundances in the ARC of AEA-treated animals together with elevated plasma AEA levels around the same time indicate a possible counter-regulatory mechanism. Nonetheless, future studies should focus on the investigation of hypothalamic orexigenes within 1 h after i.p. endocannabinoid administration and feeding with minimal exposure to stress. Moreover, future studies need to determine if higher dosages or multiple injections per day can increase feed intake in the long-term.

In accordance with the endocannabinoid-mediated short-term increase in feed intake, AEA and 2-AG treatments increased whole-body COX and metabolic HP and reduced whole-

body FOX. When analyzed independently of feed intake, COX was not different between treatment groups, whereas FOX remained lower with AEA, and 2-AG; suggesting that effects extend beyond differences in feed intake and that AEA and 2-AG suppress whole-body fat catabolism and support lipogenesis. Regarding the lipid metabolism, injected endocannabinoids attenuated the increase in plasma NEFA associated with stress-induced hypophagia and decreased concentrations of palmitic acid, palmitoleic acid, and oleic acid possibly indicating an endocannabinoid-mediated flux of free fatty acids to adipocytes for triglyceride synthesis. However, there was no effect of endocannabinoids on plasma triglyceride concentrations, or the expression of hepatic genes involved in the ECS and fat metabolism.

Overall, the obtained results detailed in this thesis offer new information about the complex and versatile ECS and lay the cornerstone for future research in dairy cows.

# Chapter 7: Summary

Summary of the PhD thesis:

**Investigations into the endocannabinoid system and endocannabinoid-mediated control of energy homeostasis in late-lactating dairy cows**

The endocannabinoid system (ECS) is a comprehensive and multifaceted system within the body of all vertebrates. It consists of G protein-coupled cannabinoid receptors, their endogenous ligands, the so-called endocannabinoids, and the corresponding synthesizing and degrading enzymes. In recent years, intensive research has provided insights into the key elements of the ECS and its vast involvement in many regulatory processes in non-ruminants, primarily rodents and humans. Several mechanisms of endocannabinoid action have been identified in terms of energy homeostasis with the regulation of feed intake and appetite, as well as the stress response and lipid and glucose metabolism. Nevertheless, numerous questions remain unanswered, especially with regard to ruminant species.

In dairy cows, targeted activation of the ECS may represent a promising therapeutic approach to overcome problems associated with the periparturient period, but the current understanding of the ECS in ruminants is insufficient. Therefore, the aim of the present work was to further characterize the fundamentals of the ECS in dairy cows and to investigate its involvement in the regulatory processes of energy homeostasis by administering the two major endocannabinoids N-arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). To achieve this aim, a series of experiments were conducted with non-pregnant, late-lactating Simmental cows.

A subset of experiments within this thesis was conducted for a better understanding of the dynamics of the circulatory endocannabinoid tone in dairy cows. Dairy cows were subjected to: (*i*) short-term feed deprivation; (*ii*) stress exposure; (*iii*) different diet compositions (low and high n6/n3 fatty acid ratio); and (*iv*) i.p. injections of AEA and 2-AG, with subsequent analysis of plasma endocannabinoid concentrations. Analyses revealed that both feed deprivation and stress exposure increase plasma 2-AG levels to varying degrees in ruminants, similar to non-ruminates, whereas plasma AEA levels remained constant. It seems that in dairy cows, 2-AG appears to be more involved in the systemic metabolic response to feed deprivation and stress exposure than AEA. Contrary to expectations, feeding a high n6/n3 diet did not increase plasma endocannabinoid concentrations in dairy cows. Systemic administration of AEA increased circulatory AEA concentrations 2.5 h after injection, while administration of 2-AG did not change plasma endocannabinoid concentrations 2.5 h after injection. The apparent

differences in the bioavailability of injected AEA and 2-AG and possible accumulation of AEA, require more detailed studies in the future.

To investigate the effects of i.p. administered endocannabinoids on feed intake and hypothalamic orexigenic signaling in dairy cows, feed intake was continuously measured by electronic registration devices and analyzed in different intervals. Analyses revealed that i.p. administered AEA and 2-AG at doses of 5 µg/kg and 2.5 µg/kg, respectively, mainly affected feed intake in the short-term within the first hour after treatment and had no long-term effect. At the end of the trial, cows were slaughtered 2 to 3 hours after i.p. endocannabinoid injection, and brain tissue was collected for immunohistochemical analysis of agouti-related peptide (AgRP), orexin A (OX-A), and cannabinoid receptor 1 ($CB_1$) expressing neurons, and for PCR analysis of AgRP, neuropeptide Y (NPY), and genes related to the ECS. The immunohistological investigation of hypothalamic orexigenes revealed, however, no neuromodulatory effect of either treatment on immunoreactivity or c-Fos activation. The mRNA gene expression of ECS-related genes in the arcuate (ARC) and paraventricular (PVN) nuclei of dairy cows was also not different between treatment groups. However, NPY and AgRP mRNA abundances were downregulated in the ARC of AEA-treated animals, which might indicate a possible counter-regulatory mechanism.

To test whether cows may benefit from injected endocannabinoids during stress in terms of stress-induced alterations in feed intake, feed intake was compared with and without exposure to stress stimuli such as social and tactile isolation, and tethering. In fact, AEA and 2-AG administration seemed to attenuate stress-induced hypophagia.

To further understand the influence of administered endocannabinoids on the level of whole-body metabolism, cows were kept in respiration chambers twice for three days, and gas exchange was recorded every 6 minutes for detailed analysis. Treatment with AEA and 2-AG increased whole-body carbohydrate oxidation (COX) and metabolic heat production (HP) and decreased whole-body fat oxidation (FOX), mirroring the endocannabinoid-mediated changes in feed intake mentioned above. An analysis independent of feed intake showed that COX was not different between treatment groups, whereas FOX remained lower with endocannabinoid treatment, suggesting that effects extend beyond individual differences in feed intake and that AEA and 2-AG indeed suppress whole-body fat catabolism and/or support lipogenesis. To gain an initial understanding of the effects of administered endocannabinoids on lipid metabolism, plasma samples were collected before and after 9 days of daily treatment and analyzed for plasma non-esterified fatty acids (NEFA) and specific plasma lipid concentrations. We demonstrated that injected endocannabinoids attenuated the increase in plasma free fatty acid concentration associated with stress-induced hypophagia. Furthermore, the analyses suggest that possibly with endocannabinoid application, free fatty acids are used to an increased extent for triglyceride synthesis in adipocytes.

In conclusion, the herein discussed results offer new and significant insights into the ECS in dairy cows. This field of research is still in its early stages; thus, much remains to be discovered including the potential for beneficial therapeutic intervention. However, this thesis and the studies published within provide a direction and serve as a foundation for future research.

Zusammenfassung der Dissertation:

**Untersuchungen zum Endocannabinoidsystem und zur endocannabinoidvermittelten Kontrolle der Energiehomöostase bei spätlaktierenden Milchkühen**

Das Endocannabinoid-System (ECS) ist ein umfassendes und vielschichtiges System im Körper aller Wirbeltiere. Es besteht aus G-Protein-gekoppelten Cannabinoidrezeptoren, ihren endogenen Liganden – den sogenannten Endocannabinoiden – und den entsprechenden synthetisierenden und abbauenden Enzymen. In den letzten Jahren hat intensive Forschung wertvolle Einblicke in die Schlüsselelemente des ECS und seine weitreichende Beteiligung an vielen Regulationsprozessen bei Nicht-Wiederkäuern, vor allem bei Nagetieren und Menschen, ermöglicht. Im Hinblick auf die Energiehomöostase mit der Steuerung der Futteraufnahme und des Appetits sowie der Stressreaktion und des Lipid- und Glukosestoffwechsels wurden viele Mechanismen der Endocannabinoidwirkung identifiziert. Dennoch blieben viele Fragen unbeantwortet, insbesondere in Bezug auf Wiederkäuer.

Bei Milchkühen könnte die gezielte Aktivierung des ECS ein vielversprechender therapeutischer Ansatz zur Bewältigung von Problemen in der Transitphase darstellen, jedoch ist das derzeitige Verständnis über das ECS bei Wiederkäuern nicht ausreichend. Ziel der vorliegenden Arbeit war es daher, die Grundlagen des ECS bei Milchkühen weiter zu charakterisieren und seine Beteiligung an den Steuerungsprozessen der Energiehomöostase durch Verabreichung der beiden Endocannabinoide N-Arachidonylethanolamid (Anandamid, AEA) und 2-Arachidonylglycerol (2-AG) zu untersuchen. Um dieses Ziel zu erreichen, wurden eine Reihe von Versuchen mit nicht trächtigen, spätlaktierenden Simmentaler Kühen durchgeführt.

Im Rahmen dieser Dissertation wurden eine Reihe von Experimenten durchgeführt, um ein besseres Verständnis der Dynamik des zirkulatorischen Endocannabinoid-Tonus bei Milchkühen zu erlangen. Milchkühe wurden (*i*) kurzzeitigem Futterentzug, (*ii*) Stressbelastung, (*iii*) verschiedenen Rationszusammensetzungen (niedriges und hohes n6/n3-Fettsäureverhältnis) und (*iv*) i.p.-Injektionen von AEA und 2-AG ausgesetzt, mit anschließender Analyse der Plasma-Endocannabinoid-Konzentrationen. Die Analysen ergaben, dass sowohl Futterentzug als auch Stressbelastung die 2-AG-Konzentration im Plasma von Milchkühen in unterschiedlichem Maße erhöhten, ähnlich wie bei Nicht-Wiederkäuern, während die AEA-Konzentration im Plasma unverändert blieb. Es scheint, dass 2-AG bei Milchkühen stärker an der systemischen Stoffwechselreaktion auf Futterentzug und Stressbelastung beteiligt ist als AEA. Anders als bei Nicht-Wiederkäuern führte die Fütterung einer n6/n3-reichen Ration bei Milchkühen nicht zu einer Erhöhung der Endocannabinoid-Konzentrationen im Plasma. Die systemische Verabreichung von AEA erhöhte 2,5 Stunden

nach der Injektion die AEA-Konzentrationen im Blutkreislauf, während die Verabreichung von 2-AG die Endocannabinoid-Konzentrationen im Plasma 2,5 Stunden nach der Injektion nicht veränderte. Die Unterschiede in der Bioverfügbarkeit von injiziertem AEA und 2-AG und die mögliche Akkumulation von AEA erfordern in Zukunft genauere Untersuchungen.

Um die Auswirkungen von i.p. verabreichten Endocannabinoiden auf die Futteraufnahme und die hypothalamische orexigene Signalgebung bei Milchkühen zu untersuchen, wurde die Futteraufnahme kontinuierlich elektronisch erfasst und in verschiedenen Intervallen analysiert. Die Analysen ergaben, dass i.p. verabreichtes AEA und 2-AG in einer Dosierung von 5 µg/kg bzw. 2,5 µg/kg hauptsächlich die Futteraufnahme kurzfristig innerhalb der ersten Stunde nach der Behandlung beeinflusste und keine Langzeitwirkung hatte. Am Ende des Versuchs wurden die Kühe 2 bis 3 Stunden nach der i.p. Endocannabinoid-Injektion geschlachtet, und es wurde Hirngewebe für die immunhistochemische Analyse von Agouti-related Peptid (AgRP), Orexin A (OX-A) und Cannabinoid-Rezeptor ($CB_1$) exprimierenden Neuronen sowie für die PCR-Analyse von AgRP, Neuropeptid Y (NPY) und Genen, die mit dem ECS in Verbindung stehen, entnommen. Aus immunhistochemischen Untersuchungen hypothalamischer Orexigene ergaben sich allerdings keine Hinweise auf eine endocannabinoidvermittelte Neuromodulation oder c-Fos-Aktivierung. Die Genexpression von ECS-assoziierten Genen im Nucleus arcuatus (ARC) und Nucleus paraventricularis (PVN) von Milchkühen auf mRNA-Ebene unterschied sich ebenfalls nicht zwischen den Behandlungsgruppen. Allerdings wurden die mRNA-Abundanzen von NPY und AgRP im ARC von AEA-behandelten Tieren herunterreguliert, was auf einen möglichen gegenregulatorischen Mechanismus hinweisen könnte.

Um zu testen, ob Kühe möglicherweise von der Verabreichung von Endocannabinoiden bei Stress im Hinblick auf stressbedingte Veränderungen der Futteraufnahme profitieren, wurde die Futteraufnahme mit und ohne Stressreize wie soziale und taktile Isolation und Anbindehaltung verglichen. Es wurde herausgefunden, dass die Verabreichung von AEA und 2-AG die stressinduzierte Hypophagie abschwächt.

Um den Einfluss der verabreichten Endocannabinoide auf den Gesamtstoffwechsel besser zu verstehen, wurden die Kühe zweimal drei Tage lang in Respirationskammern gehalten, um den Gasaustausch kontinuierlich aufzuzeichnen. Es zeigte sich, dass die Behandlung mit AEA und 2-AG zu einem Anstieg der Kohlenhydratoxidation (COX) und der metabolen Wärmeproduktion (HP) sowie zu einem Rückgang der Fettoxidation (FOX) führte, was die oben erwähnten endocannabinoidvermittelten Veränderungen der Futteraufnahme widerspiegelt. Eine von der Futteraufnahme unabhängige Analyse zeigte, dass sich die COX-Werte zwischen den Behandlungsgruppen nicht unterschieden, während die FOX-Werte unter der Endocannabinoid-Behandlung niedriger blieben, was darauf hindeutet, dass die Endocannabinoidwirkung über die Effekte auf Futteraufnahme hinausgeht, und dass AEA und

2-AG den Fettabbau unterdrücken und die Lipogenese fördern. Um ein erstes Verständnis der Auswirkungen der verabreichten Endocannabinoide auf den Fettstoffwechsel zu gewinnen, wurden vor und nach 9 Tagen täglicher Behandlung Plasmaproben entnommen und auf die Konzentration freier Fettsäuren im Plasma sowie auf weitere Plasmalipidkonzentrationen untersucht. Wir konnten zeigen, dass die injizierten Endocannabinoide den mit der stressinduzierten Hypophagie verbundenen Anstieg der Konzentration freier Fettsäuren im Plasma abschwächten. Ferner deuten die Analysen darauf hin, dass möglicherweise mit Endocannabinoid-Applikation freie Fettsäuren in erhöhtem Maße zur Triglyceridsynthese in Adipozyten verwendet werden.

Zusammenfassend lässt sich feststellen, dass die hier diskutierten Ergebnisse neue und wichtige Erkenntnisse über das ECS bei Milchkühen liefern. Dieses Forschungsgebiet befindet sich noch in der Anfangsphase, und es sind weitere Studien zum ECS bei der Milchkuh notwendig, um das therapeutische Potential von Endocannabinoiden weiter zu heben. Die vorgestellten Ergebnisse und die veröffentlichten Studien dienen jedoch als Grundlage für künftige Forschungen.

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# List of publications

## First-author publications as part of the dissertation (peer reviewed)

**van Ackern, I**., Kuhla, A. Kuhla, B. (2021):
A role for peripheral anandamide and 2-arachidonoylglycerol in short-term food intake and orexigenic hypothalamic responses in a species with continuous nutrient delivery.
Nutrients 13: 3587. DOI: 10.3390/nu13103587.

**van Ackern, I**., Wulf, R., Dannenberger, D., Tuchscherer, A., Kuhla, B. (2021):
Effects of endocannabinoids on feed intake, stress response and whole-body energy metabolism in dairy cows.
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## Co-author publications without reference to dissertation (peer reviewed)

Kuhla, B., **van Ackern, I.** (2022):
Effects of intracerebroventricular anandamide administration on feed intake and milk yield of dairy cows.
JDS Comm 3: 138-141. DOI: 10.3168/jdsc.2021-0185.

## Abstracts in proceedings and participation in conferences

**van Ackern, I**., Kuhla, B. (2019):
Effect of endocannabinoids on energy metabolism of the late lactating Simmental cow.
19th Day of the Doctoral Student, Nov 7th, 2019, Leibniz Institute for Farm Animal Biology
Abstracts p. 30.

**van Ackern, I**., Tuchscherer A., Kuhla, B. (2020):
Effect of feed deprivation on plasma endocannabinoid concentrations in dairy cows.
74. Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE) Göttingen 03.-05.03.2020
*Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie* 38, p. 63, ISBN: 978-3-7690-4113-2.

**van Ackern, I**., Tuchscherer A., Kuhla, B. (2020):
Effect of endocannabinoids on feed intake and energy metabolism of Simmental cows in established lactation.
20th Day of the Doctoral Student, Nov 12th, 2020, Leibniz Institute for Farm Animal Biology
Abstracts p. 31.

**van Ackern, I**., Kuhla, B. (2022):
Intracerebroventricular N-arachidonoylethanolamide administration increases feed intake and reduces milk yield in late lactating dairy cows – results from a pilot study.
76. Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE) Göttingen 08.-10.03.2022
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## Conflict of interest

Within the scope of this work, there are no conflicts of interest due to third-party donations.

## Declaration of independence

I hereby declare that I have prepared this thesis solely by myself. I assure that I have used only the sources and aids provided.

Berlin, den 12.07.2023

Isabel van Ackern


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