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**Original Research**

# Inhibition of endocannabinoid synthesis enzymes DAGL and NAPE-PLD transiently lowers body weight and alters glucose homeostasis during a high-fat diet challenge in mice

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## Abstract

**Background:** Cannabinoid receptor 1 inhibition poses an effective treatment strategy in obesity, but has a risk of psychiatric side effects. As high-fat diet (HFD)-feeding acutely increases expression of endocannabinoid synthesis enzymes and circulating endocannabinoid levels in mice, here we tested whether inhibition of these enzymes alleviates metabolic disturbances caused by high-fat diet feeding.

**Methods:** C57BL/6J mice received daily intraperitoneal injections with a NAPE-PLD inhibitor (LEI-401), DAGL inhibitor (DH-376), or vehicle for 1 week while on a HFD. An extra group of vehicle-treated mice was maintained on regular chow diet.

**Results:** Both inhibitors effectively lowered blood and brain levels of endocannabinoids after 1 week of treatment. DH-376 reduced plasma insulin levels compared with vehicle (−53%) already within 2 h after the first dose. In contrast, LEI-401 did not change insulin or glucose levels. Both inhibitors suppressed caloric intake during the first day of treatment (−25% and −21%, respectively). In addition, LEI-401 elevated carbohydrate oxidation. The combined effect was a 2.0 and 1.6 g lower body weight after 24 h, respectively. Nevertheless, after 1 week body weight was no longer different between the HFD-fed groups. Moreover, DH-376 increased brown adipose tissue weight and reduced insulin-stimulated glucose uptake.

**Conclusions:** DAGL and NAPE-PLD inhibition effectively lower levels of endocannabinoids and related bioactive lipids. Both inhibitors caused a transient reduction in food intake and body weight, but also led to alterations in glucose homeostasis. The long-term effects of endocannabinoid biosynthesis inhibitors on (cardio)metabolic health remains to be investigated.

**Keywords:** adipose tissue, adipose tissue, brown, atherosclerosis, cardiometabolic disease, endocannabinoids, glucose, liquid chromatography-mass spectrometry, metabolism, obesity

### Significance

In this paper, we demonstrate that during a high-fat diet challenge, inhibition of the endocannabinoid system through inhibition of the endocannabinoid synthesis enzymes NAPE-PLD and DAGL is an effective strategy to lower blood and brain levels of endocannabinoids and related bioactive lipids produced within the corresponding syn-thesis pathways. Importantly, we found that both NAPE-PLD and DAGL inhibition acutely lower food intake coinciding with a transient reduction in body weight. This is significant given the renewed interest in endocannabinoid system inhibition for the treatment of obesity, as is currently being investigated in phase 2 clinical trials (*eg*, nimacimab; monlunabant).

## Introduction

Over the past decades the endocannabinoid system has been uncovered as a central regulator of metabolism, which makes it an interesting target for treatment of obesity and obesity-related disorders. The endocannabinoid system comprises of lipid-based endocannabinoids, their receptors, and the enzymes responsible for the synthesis and degradation of endocannabinoids. The 2 most prominent endocannabinoids are anandamide (arachidonoyl ethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG). AEA is produced from *N*-arachidonoyl phosphatidylethanolamine after hydrolysis by the enzyme *N*-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD). This enzyme

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also synthesizes many other endogenous bioactive lipids called *N*-acylethanolamines (NAEs). 2-AG is synthesized in a separate pathway by hydrolysis of *sn*-2-arachidonate-containing diacylglycerols by the enzyme diacylglycerol lipase (DAGL). Endocannabinoids are produced and released on demand, and 2-AG and AEA are rapidly degraded by the enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) to produce arachidonic acid (AA) and either glycerol or ethanolamine, respectively.[1-3]

Both AEA and 2-AG are ligands for the cannabinoid type 1 and type 2 receptors (CB1R; CB2R). While the CB2R is primarily expressed by immune cells[4] and is generally known to regulate immune responses, the CB1R is widely expressed in the central nervous system where it plays a role in modulating neurotransmission.[5] The CB1R is also expressed in peripheral metabolic tissues including white adipose tissue (WAT) and brown adipose tissue (BAT) where it controls, among others, lipid oxidation as we reviewed previously.[6] It is also expressed in the liver and skeletal muscle where it regulates glucose metabolism.[7,8]

Circulating levels of AEA and 2-AG are elevated in humans living with obesity,[9] and in humans circulating 2-AG levels positively correlate with measures of obesity including BMI, body fat percentage, circulating triglyceride levels and measures of glucose resistance.[9-12] Indeed, NAPE-PLD has been associated with food and reward-driven behaviors and energy balance in mice.[13] Furthermore, mice genetically deficient for the CB1R are protected against high-fat diet (HFD)-induced obesity[14] and systemic inverse agonism of the CB1R by rimonabant was shown to significantly lower body weight in both mice[15] and humans.[16] In fact, rimonabant was one of the first anti-obesity drugs to reach the market, and was successful in lowering body weight, reducing waist circumference and improving atherogenic dyslipidemia.[17] However, serious psychiatric side effects, likely mediated via accumulation of rimonabant in the brain in combination with a long receptor residence time, led to its withdrawal 2 years after its introduction. Since then, strategies have focused on the identification of new allosteric and peripherally restricted CB1R antagonists, currently in phase 2 trials for the treatment of obesity (eg, Nimacimab[18]; Monlubant[19]).

Interestingly, circulating AEA and 2-AG levels in mice rapidly increase upon HFD feeding, even before weight gain becomes apparent (*ie,* after 1 or 4 weeks, respectively).[20,21] Furthermore, we observed a higher expression of endocannabinoid synthesis enzymes within adipose tissue upon short-term HFD feeding.[20] These observations support the hypothesis that inhibition of endocannabinoid synthesis enzymes themselves may benefit metabolic health. We have recently developed and characterized LEI-401 as an inhibitor for NAPE-PLD,[22] as well as DH-376 as an inhibitor for DAGL,[23] of which we have previously shown to lower fasting-induced refeeding.[24] Here, we therefore investigated the short-term metabolic effects of NAPE-PLD and DAGL inhibitors during a 1-week HFD challenge in mice.

## Materials and methods

### Animals and intervention

All mouse experiments had received approval from the National Committee for Animal Experimentation of the Netherlands ("Centrale Commissie Dierproeven"). Throughout the entire experiment, 8 weeks-old male C57Bl/6J mice were individually housed in calorimetric home cages (Promethion line, Sable Systems International) at 22 °C under a 12h:12 h light:dark cycle (lights on at 7:00 AM clock time). Access to food was limited to the dark phase (ie, 7:00 PM to 7:00 AM clock time). After 3 days of acclimatization, mice were assigned to 1 of 4 groups that were balanced for body weight and body composition using RandoMice version 1.0.3.[25] Three groups were switched to a HFD containing 45 kJ% fat (19.3 MJ metabolizable energy·kg$^{-1}$; #824053, Tecnilab-BMI BV) and received a daily intraperitoneal injection (6.7 µL·g$^{-1}$) with vehicle (DMSO: Tween-80:$H_2O$ in a ratio 1:1:8), a NAPE-PLD inhibitor (LEI-401; 30 mg·kg$^{-1}$) or a DAGL inhibitor (DH-376; 50 mg·kg$^{-1}$). In addition, one group was maintained on a chow diet (11.2 MJ metabolizable energy·kg$^{-1}$) and received a daily intraperitoneal injection with vehicle. Intraperitoneal administration of test substance was done between 5:00 PM and 6:00 PM clock time, unless stated otherwise. After an intervention period of 7 days in total, *in vivo* glucose uptake upon insulin stimulation was determined and mice were killed by cervical dislocation.

### Indirect calorimetry

During the intervention period, food intake, $O_2$ consumption ($VO_2$) and $CO_2$ production ($VCO_2$) were recorded at 5 min intervals in the calorimetric home cages (Promethion line, Sable Systems International). $VO_2$ and $VCO_2$, as measured between 7:00 PM on the first day of intervention and 7:00 AM on the last day of intervention, were used to estimate the respiratory exchange ratio (RER), fat oxidation rate, carbohydrate oxidation rate, and total energy expenditure.

### Body weight and body composition

Body weight was determined daily before injection with the test substance by placing the mouse on a regular weighing scale (440-43N; KERN). Body composition was determined using an EchoMRI-100 (EchoMRI) 3 days before the start, and 1 day before the end of the intervention period.

### Plasma glucose and insulin levels

On the first and last day of the intervention period, mice received the intraperitoneal injection with the test substance at 11:00 AM to collect tail vein blood at 1:00 PM (based on[22,23]). From these blood samples, plasma was obtained for colorimetric measurement of glucose levels (10786; HUMAN) and determination of insulin levels using an Ultra-Sensitive Mouse Insulin ELISA Kit (90080, Crystal Chem).

### In vivo glucose uptake upon insulin stimulation

Immediately after the collection of tail vein blood on the last day of the intervention period, mice were intravenously injected with 0.5 U·kg$^{-1}$ body weight insulin (NovoRapid, Novo Nordisk) and 1 µCi 2-[1-$^{14}$C]-deoxy-D-glucose (Perkin Elmer). After 15 min (*ie,* in line with[26]), mice were killed by cervical dislocation, heart puncture blood was collected, and interscapular BAT (iBAT), subscapular BAT (sBAT), the spleen, gonadal WAT (gWAT), subcutaneous WAT (sWAT), the heart and the liver were collected and weighed. Parts of organs (max 250 mg) were dissolved overnight at 56 °C in 0.5 mL Solvable (PerkinElmer), after which 2-[1-$^{14}$C]-deoxy-D-glucose-derived radioactivity was determined using a liquid scintillation counter

(Tri-Carb 2910 TR; PerkinElmer) after addition of 5 mL Ultima Gold (PerkinElmer). Data are expressed as percentage of the injected dose per gram of the corresponding wet tissue.

### Endocannabinoid measurements in plasma and tissue samples

In plasma obtained after heart puncture, relative levels of endocannabinoids and related endogenous bioactive lipids (*ie*, AEA, palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), docosahexaenoyl ethanolamide (DHEA), stearoyl ethanolamide (SEA), linoleyl ethanolamine (LEA), alpha-linolenoyl ethanolamide (α-LEA), dihomo-γ-linolenoyl ethanolamide (DGLEA), pentadecenoyl ethanolamide (PDEA), and palmitoleoyl ethanolamide (POEA), 2-AG, 2-linoleoyl glycerol (2-LG), 2-oleoyl glycerol (2-OG) and AA) were extracted and measured by LC-MS/MS as described before.[27]

In addition, the same lipids were extracted from approximately 15-60 mg tissue of the brain, quadriceps muscle, liver, gWAT, sWAT and iBAT, and measured by LC-MS/MS following previously described protocols.[28] Briefly, homogenized tissues were extracted using organic solvents (methanol for all the samples; a liquid-liquid extraction based on n-hexane and methanol/water for iBAT) containing a mixture of deuterated internal standards. After centrifugation, the supernatants were dried and reconstituted in a solvent matching the starting conditions of the chromatographic gradient. A tissue specific quality control (QC) sample was made by pooling a small aliquot of all the samples per tissue type. The QC was analyzed periodically along the analytical run. Data was only considered for statistical significance if the fold change also exceeded 2 times the relative standard deviation (RSD) of the QC pool as presented in Table S1. The LC-MS/MS system was an HPLC system from Shimadzu coupled to a Sciex QTrap MS/MS system equipped with electrospray ionization (ESI). The chromatographic separation was achieved using a Kinetex 2.6 μm C18 100 Å size LC column 50 × 2.1 mm coupled to a C8 precolumn of 2.1 mm. All the LC-MS/MS parameters including the multiple reaction monitoring (MRM) transitions can be obtained from a previous dedicated protocol.[28] Analytical characteristics of the LC-MS/MS method (*ie*, accuracy, recovery, precision, linearity and limits of detection (LOD) and quantification (LOQ)) are presented in Tables S2-S6.

### Statistical analyses

Differences between the treatment groups were determined by ANOVA and after Dunett's post-hoc analysis, compared with the HFD-vehicle group. $P$ values $< .05$ were considered statistically significant.

## Results

### NAPE-PLD and DAGL inhibition effectively lower endocannabinoid levels

Lean male C57Bl/6J mice were switched from a regular chow diet to a HFD and received a daily intraperitoneal injection with either the NAPE-PLD inhibitor LEI-401, the DAGL inhibitor DH-376, or vehicle for 7 days. In addition, a reference group was included which received vehicle treatment while kept on chow diet. For clarity and simplicity, we will refer to the vehicle groups as "HFD-vehicle" or "chow-vehicle", from this point onwards.

We employed LC-MS/MS to investigate the direct effects of LEI-401 and DH-376 on plasma and tissue levels of endocannabinoids and related endogenous bioactive lipids. Similar to our previous observations,[20] one week of HFD feeding increased plasma levels of AEA but not 2-AG (Figure 1A). In addition, we found plasma levels of OEA and DHEA to be increased upon HFD feeding (Figure 1A). Within the brain, we could not observe any changes in endocannabinoid levels upon HFD feeding (Figure 1B). In peripheral tissues, however, we observed varying effects. For example, HFD-feeding led to increased AEA levels within iBAT, but decreased levels of 2-AG in sWAT (Figure 1C-G).

Both LEI-401 and DH-376 appeared effective at lowering the levels of endocannabinoids and related lipids that are produced within the synthesis pathways of the corresponding enzymes. Specifically, LEI-401 treatment strongly lowered the levels of AEA, as well as PEA, OEA, DHEA, SEA, LEA, DGLEA and POEA in the brain (Figure 1B). On the other hand, DH-376 strongly lowered the levels of 2-AG and 2-OG compared with HFD-vehicle in the brain (Figure 1B), and lowered the plasma and brain levels of AA, which is the main breakdown product of endocannabinoids (Figure 1A, B). DH-376 furthermore reduced the levels of the same lipid species as well as 2-LG within muscle (Figure 1C), and lowered 2-LG and 2-OG within the liver (Figure 1D). Surprisingly, DH-376 lowered the levels of AEA but not of 2-AG within iBAT (Figure 1G). All results described above are visualized in a heat map (Figure 1H).

### DAGL inhibition acutely lowers plasma insulin levels

Endocannabinoid signaling has been implicated in acute insulin secretion from pancreatic β-cells, and therefore we measured fasted plasma levels of glucose and insulin 2 h after the first injection with the inhibitors, and 2 h after the last injection on day 7. Immediately after the first injection, DAGL inhibition by DH-376 approximately halved fasting plasma insulin levels without affecting glucose levels, while NAPE-PLD inhibition by LEI-401 did not affect either of these levels (Figure 2A, B). After 1 week, HFD-feeding led to increased plasma glucose and insulin levels, indicating the development of insulin resistance (Figure 2C, D). At this point, plasma insulin levels in DH-376-treated mice were even further lowered to one-fourth of that of mice in the HFD-vehicle group, again with no change in plasma glucose levels (Figure 2D), while NAPE-PLD inhibition again did not affects either of these levels.

### NAPE-PLD and DAGL inhibition transiently lower food intake and transiently lower HFD-induced body weight gain

In the first 24 h after the diet switch, food intake in the HFD-vehicle group was higher than in the chow-vehicle group (average 5.6 g/mouse *vs.* average 3.6 g/mouse; Figure 3A), corresponding with an almost 3-fold increased intake of metabolizable energy (Figure 3B). Interestingly, LEI-401 and DH-376 treatment partially prevented the initial HFD-induced increase in food intake, causing caloric energy intake to be 21% and 25% lower than in the HFD-vehicle group (Figure 3A, B). However, those effects were only temporarily and throughout the remainder of the intervention, mice within these treatment groups seemed to compensate for this initial drop in energy intake as average food intake

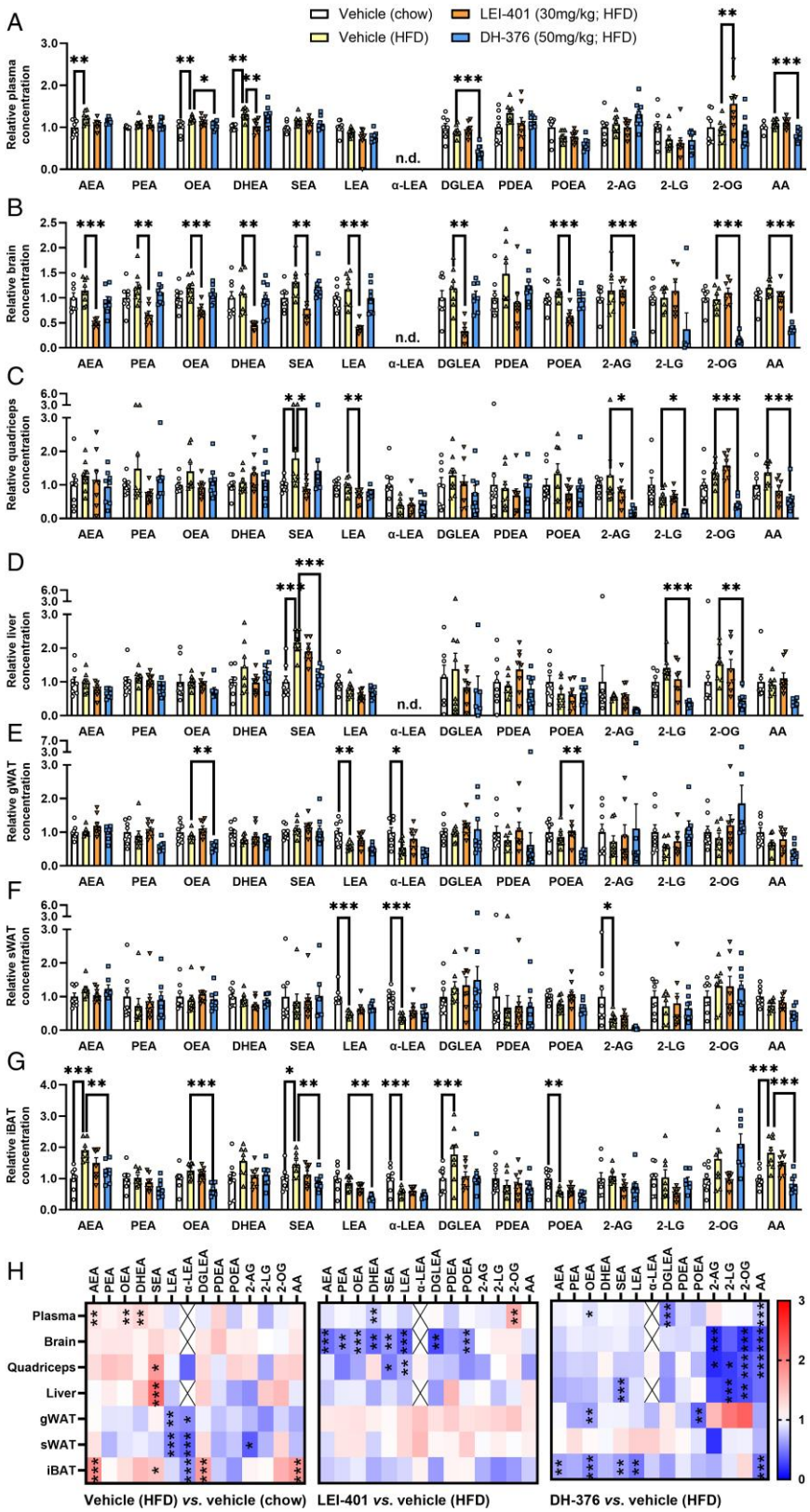


**Figure 1.** NAPE-PLD and DAGL inhibition effectively lower endocannabinoid levels. Lean male C57Bl/6J mice were switched from a regular chow diet to a HFD and received a daily intraperitoneal injection with vehicle, a NAPE-PLD inhibitor (LEI-401; 30 mg·kg$^{-1}$) or a DAGL inhibitor (DH-376; 50 mg·kg$^{-1}$). In addition, a reference group was included which received vehicle treatment while kept on chow diet. After 7 days of treatment, mice were killed. In (A) plasma obtained after heart puncture and in the (B) brain, (C) quadriceps, (D) liver, (E) gonadal white adipose tissue (gWAT), (F) subcutaneous WAT (sWAT) and (G) interscapular brown adipose tissue (iBAT), relative levels of endocannabinoids and related endogenous bioactive lipids were extracted and measured using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). (H) Changes in levels of endocannabinoids and related endogenous bioactive lipids as depicted in A-G were additionally visualized in heatmaps. Data are presented as mean ± SEM. $n = 8$ per group. *$P < .05$, **$P < .01$, ***$P < .001$ vs. vehicle-HFD. 2-LG, 2-linoleoyl glycerol; 2-OG, 2-oleoyl glycerol; AA, arachidonic acid; AEA, arachidonoyl ethanolamide; DGLEA, dihomo-γ-linolenoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide; LEA, linoleyl ethanolamine; n.d., not determined; OEA, oleoyl ethanolamide; PDEA, pentadecenoyl ethanolamide; PEA, palmitoyl ethanolamide; POEA, palmitoleoyl ethanolamide, 2-AG, 2-arachidonoyl glycerol; SEA, stearoyl ethanolamide; α-LEA, alpha-linolenoyl ethanolamide.

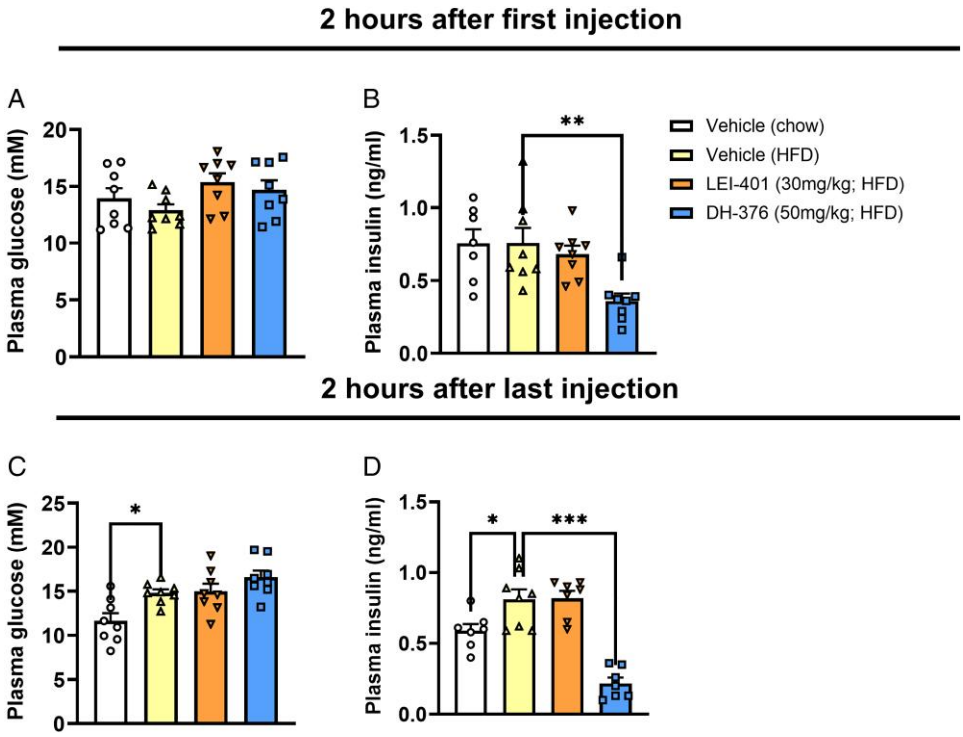


**Figure 2.** DAGL inhibition acutely lowers plasma insulin levels. Lean male C57Bl/6J mice were switched from a regular chow diet to a HFD and received a daily intraperitoneal injection with vehicle, a NAPE-PLD inhibitor (LEI-401; 30 mg·kg$^{-1}$) or a DAGL inhibitor (DH-376; 50 mg·kg$^{-1}$). In addition, a reference group was included which received vehicle treatment while kept on chow diet. On the (A-B) first and (C-D) last day of the intervention period, plasma (A, C) glucose and (B, D) insulin were determined. Data are presented as mean ± SEM. $n = 8$ per group. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs. vehicle-HFD.

throughout the entire intervention period did not differ from the HFD-vehicle group (Figure 3C). When comparing total energy expenditure as determined by indirect calorimetry throughout the study, no differences were found between the groups (Figure 3D). As expected, HFD feeding led to a decrease in the RER compared with chow feeding (Figure 3E), and a corresponding switch from carbohydrate oxidation (Figure 3F) to fat oxidation (Figure 3G). Interestingly, LEI-401, but not DH-376, partially prevented this switch in substrate utilization (Figure 3E-G).

The combined effect was respectively a 1.6 and 2.0 g lower body weight in LEI-401 and DH-376 treated mice than in HFD-vehicle treated mice after 24 h of treatment. Nevertheless, body weight did not differ from the HFD-vehicle group anymore after 7 days of treatment (Figure 3H).

At the end of the treatment period, mice in the HFD-vehicle group overall had a higher body fat mass and comparable lean mass compared with chow-vehicle mice (Figure 3I). LEI-401 and DH-376 did not affect body composition (Figure 3I). In line with these results, 1 week of HFD feeding led to fat accumulation in gWAT reflected in increased tissue weight compared with chow-vehicle, and LEI-401 and DH-376 treatment did not affect gWAT or sWAT weights compared with the HFD-vehicle group (Figure 3J-K). DH-376-treated mice, however, did show a higher iBAT weight (+78%; Figure 3J).

### DAGL inhibition lowers insulin-stimulated glucose uptake within BAT

To investigate whether we could link changes in endocannabinoid levels within blood and tissues with changes in glucose metabolism, we injected mice intravenously with a mixture of insulin and tracer amounts of 2-[1-$^{14}$C]-deoxy-D-glucose 15 min prior to organ collection. Similar to our previous report,[26] 1 week of HFD feeding strongly decreased insulin-stimulated glucose uptake by iBAT (11.7 *vs.* 19.2% of injected dose/gram) and sBAT (12.9 *vs.* 20.6%), as well as sWAT (1.0 *vs.* 1.8%) (Figure 3L). LEI-401 did not affect the insulin-stimulated glucose uptake by BAT or WAT, but modestly increased the uptake by the liver compared with HFD-vehicle (3.3 *vs.* 2.2%; Figure 3L). Unexpectedly, we found DH-376 to even further lower insulin-stimulated glucose uptake by iBAT (to 5.8%) and sBAT (to 6.4%), and furthermore to lower the uptake by pVAT compared with HFD-vehicle (5.4% *vs.* 10.8%) (Figure 3L).

## Discussion

Numerous studies have revealed the endocannabinoid system as a regulator of metabolism (*eg*,[6-8]), and endocannabinoid levels positively correlate with various measures of obesity in humans.[9-12] In this context, we previously observed higher circulating AEA and 2-AG levels in mice upon short-term HFD feeding, coinciding with higher expression of endocannabinoid synthesis enzymes within adipose tissue.[20] In the current study, we therefore investigated the effects of inhibition of the endocannabinoid synthesis enzymes NAPE-PLD and DAGL during a HFD challenge in mice.

We quantified a multitude of endocannabinoids and related bioactive lipids within blood, the brain, and peripheral metabolic tissues. NAPE-PLD inhibition prevented the HFD-induced increase in plasma AEA levels. Strikingly in the brain, both NAPE-PLD and DAGL inhibition consistently

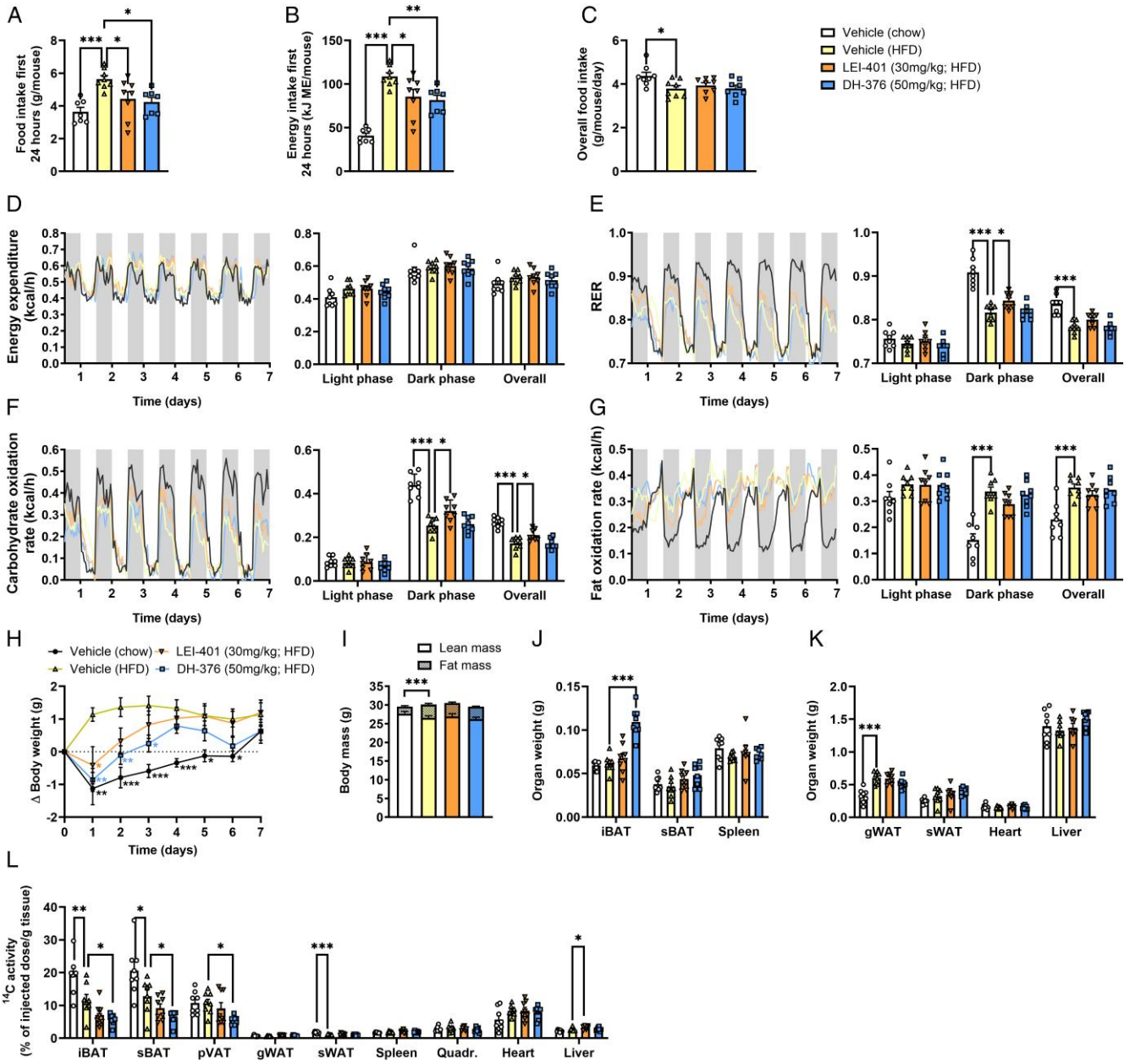


**Figure 3.** NAPE-PLD and DAGL inhibition transiently lower food intake and body weight. Lean male C57Bl/6J mice were switched from a regular chow diet to a HFD and received a daily intraperitoneal injection with vehicle, a NAPE-PLD inhibitor (LEI-401; 30 mg·kg$^{-1}$) or a DAGL inhibitor (DH-376; 50 mg·kg$^{-1}$). In addition, a reference group was included which received vehicle treatment while kept on chow diet. (A) Food intake was measured during the first 24 hours and (B) expressed as total metabolizable energy (ME)-intake per mouse. In addition, (C) food intake as measured daily throughout the entire intervention period was averaged per mouse per day. Throughout the entire experiment, mice were individually housed in calorimetric home cages to determine $O_2$ consumption and $CO_2$ production from which (D) energy expenditure, (E) the respiratory exchange ratio (RER), (F) carbohydrate oxidation rate and (G) fat oxidation rate was estimated. (H) Body weight was determined daily, and after 7 days of treatment, (I) body composition was determined, (J-K) organs were weighed, and (L) insulin-stimulated uptake of 2-[1-$^{14}$C]-deoxy-D-glucose was determined and expressed as per gram tissue. (A-C; H-L) Data are presented as mean ± SEM. (D-G) Data are plotted as average per hour over time; averages of the light and dark phase, and overall averages are presented as mean ± SEM. $n = 8$ per group. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs. vehicle-HFD. gWAT, gonadal white adipose tissue; iBAT, interscapular brown adipose tissue; pVAT, perivascular adipose tissue; Quadr., quadriceps muscle; sBAT, subscapular brown adipose tissue; sWAT, subcutaneous white adipose tissue.

lowered levels of the lipids in their respective pathways, thereby confirming target modulation as was previously reported.[22,23] Castel *et al.*[13] indeed recently showed in mice that NAPE-PLD specifically within the ventral tegmental area is required for the synthesis of NAEs, thereby playing a critical role in reward-driven behaviors. Of note, AEA levels within the various metabolic tissues were not apparently decreased, suggesting that other biosynthetic pathways are responsible for the AEA production[29] or, alternatively, that the NAPE-PLD inhibitor in peripheral tissues is less effective potentially due to higher substrate or bile acid levels, or due to tissue-specific distribution of the compound. Interestingly enough, others have previously demonstrated that adipocyte-specific ablation of *Napepld* in mice results in impaired adaptation to cold and decreased browning markers in WAT, but without changes in AEA levels within the tissue

itself.[30] They did, however, observe large changes in microbiome composition. It would thus be interesting to investigate the effects of the bioactive lipids that we found to be affected within WAT and BAT on metabolic health and microbiome composition in future experiments. We did not observe an increase in plasma 2-AG levels after 1 week of HFD-feeding. This is in line with our previous observation where we observed elevated plasma 2-AG levels only after prolonged HFD-feeding,[20] which may be related to a delayed increase in *Daglβ* expression within the adipose tissues. With regards to DAGL inhibition we could observe lowered 2-AG levels in muscle tissue besides in the brain. Given that 2-AG has been associated with decreased myoblast proliferation and differentiation at least *in vitro*,[31] it would be interesting to monitor the potential beneficial effects of long-term DAGL inhibition on muscle mass and function in future experiments.

In the current study, we found that DAGL inhibition lowered plasma insulin levels. Given the acute nature of this effect, we interpret that this is caused by a direct effect of the inhibitor on the pancreas. Indeed, direct relationships between the endocannabinoid system and pancreatic β cells have been described. For example, both 2-AG and AEA have been shown to lower insulin release by reducing intracellular calcium concentration within murine pancreatic β cells through CB1R[32] and CB2R activation.[33] In mouse β cells, endocannabinoids furthermore counteract glucagon-like peptide-1 (GLP-1)-mediated cAMP synthesis and subsequent insulin release via CB1R-mediated stimulation of $G_{i/o}$.[34] Elevated DAG levels in β cells as a consequence of DAGL inhibition may also affect insulin secretion via activation of protein kinase C.[35] Indeed, conflicting effects have also been described. For example, Bermúdez-Silva *et al.*[32] showed that that 2-AG and AEA stimulate insulin release in isolated human islets as mediated via the CB1R. While the lowered insulin levels as observed in the current study typically suggest an improved insulin-sensitivity, we found that DAGL inhibition did not increase but rather further decreased the insulin-stimulated glucose uptake by BAT. In theory it could be possible that the insulin that we administered alongside glucose was insufficient to overcome the strongly reduced insulin levels caused by DAGL inhibition, thus resulting in a net decrease in glucose uptake by BAT compared with controls, despite improved insulin sensitivity. However, this observation warrants further investigation in future studies.

We found that both NAPE-PLD and DAGL inhibition acutely lower food intake, coinciding with a transient reduction in body weight. These effects are in line with previous observations using inhibitors of CB1R by us,[24,36,37] and others (eg,[38,39]), suggesting that reduced CB1R signaling is responsible for this effect we observed. CB1R expressed in the brain may mediate this effect by affecting palatability of food (as reviewed in[40]). It may also be mediated via CB1R expressed peripherally. For example, CB1R signaling in the vagus nerve has been implicated in affecting food intake in mice,[41] and mice with a CB1R deficiency specifically in the adipose tissue have a lower caloric intake, possibly via retrograde signaling of CB1R stimuli on adipocytes towards the brain.[42] However, the question remains why CB1R inhibition effectively lowers body weight, as is currently also being demonstrated in phase 2 clinical trials (eg,[18,19]), while we could only observe a transient reduction in body weight upon NAPE-PLD and DAGL inhibition. Since the therapeutic window for intervention after 7 days was relatively small, it may still be possible that a reduction in body weight will become visible upon long-term treatment with the inhibitors. This may also be true for reductions in endocannabinoid levels given our previous observation that both circulating AEA and 2-AG levels rise even further with prolonged HFD feeding.[20] It may also be interesting to investigate the effects of the inhibitors when animals are fed a diet with an even higher caloric content. Future studies may thus focus on long-term effects, and on determining if other functions of the relevant endocannabinoids and related bioactive lipids, *ie,* functions that are not mediated via the CB1R (eg, as reviewed in[43]), may be involved. It will also be critical to address any potential psychiatric side effects of suppressing endogenous AEA or 2-AG production given the brain penetrance of the selective inhibitors used here and previous experience with the inverse CB1R agonist rimonabant. In addition, we recommend for future short-term studies to take into account any stress caused by the daily intraperitoneal injections, which was probably the reason for the initial drop in body weight in the chow-vehicle mice, for example by habituating the animals to daily injections before start of treatment.

Taken together, both NAPE-PLD inhibition by LEI-401 and DAGL inhibition by DH-376 effectively lower levels of endocannabinoids and related bioactive lipids, and resulted in a transient initial reduction in food intake and body weight, resembling previous observations with CB1R inhibitors. However, the compounds also led to alterations in glucose homeostasis. It would be interesting to investigate long-term effects of endocannabinoid biosynthesis inhibitors on (cardio)metabolic health.

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## Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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## Authors' contributions

Robin van Eenige (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Investigation [lead], Methodology [equal], Validation [equal], Visualization [lead], Writing—original draft [lead], Writing—review & editing [equal]), Elena Sánchez-López (Data curation [equal], Investigation [equal], Methodology [equal], Validation [equal], Writing—review & editing [equal]), Anna Hoekstra (Data curation [equal], Investigation [equal], Methodology [equal], Writing—review & editing [equal]), Zhixiong Ying (Investigation [equal], Writing—review & editing [equal]),

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## Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics statement

The current study does not involve research with human subjects; therefore the Declaration of Helsinki is not applicable. All mouse experiments had received approval from the National Committee for Animal Experimentation of the Netherlands ("Centrale Commissie Dierproeven").

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