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AMP-activated kinase and the endogenous endocannabinoid system might contribute to antinociceptive effects of prolonged moderate caloric restriction in mice

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Abstract

Background: Caloric restriction is associated with broad therapeutic potential in various diseases and an increase in health and life span. In this study, we assessed the impact of caloric restriction on acute and inflammatory nociception in mice, which were either fed ad libitum or subjected to caloric restriction with 80% of the daily average for two weeks.

Results: The behavioral tests revealed that inflammatory nociception in the formalin test and in zymosan-induced mechanical hypersensitivity were significantly decreased when mice underwent caloric restriction. As potential mediators of the diet-induced antinociception, we assessed genes typically induced by inflammatory stimuli, AMP-activated kinase, and the endocannabinoid system which have all already been associated with nociceptive responses. Zymosan-induced inflammatory markers such as COX-2, TNFα, IL-1β, and c-fos in the spinal cord were not altered by caloric restriction. In contrast, AMPKα2 knock-out mice showed significant differences in comparison to C57BL/6 mice and their respective wild type littermates by missing the antinociceptive effects after caloric restriction. Endocannabinoid levels of anandamide and 2-arachidonyl glyceroldetermined in serum by LC-MS/MS were not affected by either caloric restriction alone or in combination with zymosan treatment. However, cannabinoid receptor type 1 expression in the spinal cord, which was not altered by caloric restriction in control mice, was significantly increased after caloric restriction in zymosan-induced paw inflammation. Since increased cannabinoid receptor type 1 signaling might influence AMP-activated kinase activity, we analyzed effects of anandamide on AMP-activated kinase in cell culture and observed a significant activation of AMP-activated kinase. Thus, endocannabionoid-induced AMP-activated kinase activation might be involved in antinociceptive effects after caloric restriction.

Conclusion: Our data suggest that caloric restriction has an impact on inflammatory nociception which might involve AMP-activated kinase activation and an increased activity of the endogenous endocannabinoid system by caloric restriction-induced cannabinoid receptor type 1 upregulation.

Keywords

Nociception, inflammation, caloric restriction, AMP-activated kinase, endocannabinoids, cannabinoid receptor type 1

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Introduction

It is well established that lifespan as well as the progress of different diseases can be positively influenced by dietary changes.[1] This is particularly due to the fact that a number of diseases are associated with obesity and overweight which show increasing incidences worldwide. Accordingly, fasting or caloric restriction (CR) and

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weight loss have been related to improvement of, for example, hypertension or diabetes type 2, respectively.[2,3] In addition, a number of studies indicate that CR is associated with antinociceptive effects. Mice fed 30%–60% of their daily average food quantity showed reduced responses in models of acute thermal and formalin-induced inflammatory nociception.[4,5] Alternate day fasting revealed antinociceptive effects towards acute thermal noxious stimulation and acetic acid-induced visceral nociception.[6] Furthermore, clinical studies on fasting have shown beneficial pain-relieving effects for patients with rheumatoid arthritis or fibromyalgia.[7–9] However, the detailed molecular mechanisms involved in CR-induced antinociception are still not completely elucidated, although a number of potential signaling pathways have already been uncovered. These mechanisms include changes in the endogenous opioid system after CR or fasting[6,10,11] and decreases of glutamate receptors,[12] inflammatory mediators, or reactive oxygen species.[13]

Activation of AMP-activated kinase (AMPK) has also been reported after CR of 20%–40% for four weeks, 40% for eight weeks or progressive CR from 10%–30% over periods of three to seven weeks, respectively.[9,14–16] The latter CR regimen was associated with a reduced acute nociceptive response in the Hot Plate test. Furthermore, there is evidence that endocannabinoids, effectors of an endogenous neuromodulatory system, are regulated in the nervous system during food restriction. After drastic CR to 20% of normal daily intake in rats or fasting in mice, levels of two endogenous cannabinoids, anandamide (AEA), and 2-arachidonoyl glycerol (2-AG), significantly increased in specific brain regions.[17] In addition, it has been shown that expression of the corresponding receptors, predominantly cannabinoid receptor type 1 (CB1 receptor), is also influenced by dietary conditions.[18] Since activation of AMPK and the endocannabinoid system are involved in reduced nociception,[19,20] it might be suggested that they represent a part of the physiological pathways that are activated upon CR and might, thus, contribute to antinociceptive effects. A number of publications showed evidence that cannabinoids contribute to AMPK activation and thereby exert central and peripheral metabolic and cardiac effects.[21–23] In contrast, other publications revealed reduced AMPK activity after activation of the endocannabinoid system[24] or AMPK as upstream effector of the endocannabinoid system.[25] However, in the context of pain and CR, this interconnection is only sparsely studied. Since drastic CR as well as fasting, as performed in the studies mentioned above, might quickly induce malnutrition, a moderate reduction of caloric intake might be a more favorable approach to activate these systems. In this study, we investigated effects of less intense but prolonged CR on the nociceptive response in mouse models of acute and inflammatory nociception. Furthermore, we assessed several potential mediators including inflammatory genes, AMPK as well as endocannabinoids and their receptors which might contribute to effects on nociception.

Materials and methods

Animals

Male *C57BL/6* mice were obtained from Harlan Winkelmann, Germany, at the age of 6–8 weeks. Homozygous $AMPK\alpha2^{-/-}$-mice with a *C57BL/6* background were kindly provided by Prof. Benouit Viollet, France. These mice lack exon C of the AMPK gene which encodes the catalytical subunit of AMPKα2 resulting in kinase inactivation. $AMPK\alpha2^{-/-}$ mice are viable, fertile, showed no apparent differences to their wild type littermates and have a normal life span.[26] For behavioral experiments, $AMPK\alpha2^{-/-}$-mice were backcrossed with *C57BL/6* wild type mice. Heterozygous offspring were then further bred to gain wild type and $AMPK\alpha2^{-/-}$ littermates. One group of mice received food ad libitum, a second group of mice was subjected to CR with 80% of their average daily food quantity for two weeks. To determine the average food consumption per day, control and CR mice were placed in individual housing and received a defined amount of food. After 24 h, the remaining food pellets were weighed, and the average daily food quantity as well as 80% of the value was calculated for each mouse. Then daily portions for each mouse were weighed out and prepared. Before, during and after the two weeks of dieting, mice were weighed regularly in order to control weight loss. During the entire two weeks of dieting, mice were kept in individual housing to ensure that each mouse gets the calculated 80% of its average daily food quantity. To reduce social stress by separate housing, mice cages were kept closely to each other to allow the mice to see each other. After two weeks of CR, both control and CR group underwent behavioral testing and were subsequently sacrificed at the indicated time points. All experiments were performed at the same time of day to avoid circadian variations. Spinal cords were dissected out, immediately frozen in liquid nitrogen and then stored at −80°C until further preparation. In all experiments, the ethical guidelines for investigations in conscious animals were obeyed, and the procedures were approved by the local Ethics Committee for Animal Research (Regierungspräsidium Darmstadt, F95/53). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Behavioral testing

Motor coordination (Hanging wire test). Motor coordination was assessed by placing mice on a metal grid which was turned upside down and held in this inverted position for 90 s (latency period). If mice were able to remain on four limbs in the hanging position for the whole observation period, their motor coordination was rated as intact.

Mechanical sensitivity (Dynamic Plantar Test). Paw withdrawal latency (PWL) to mechanical stimulation was assessed with an automated testing device consisting of a steel rod that was pushed against the plantar surface of the paw with increasing force until the paw was withdrawn (Dynamic Plantar Aesthesiometer, Ugo Basile, Varese, Italy). The maximum force was set at 5 g to prevent tissue damage, and the ramp speed was 0.5 g/s. The cut off time was 20 s. Mice were placed in test cages with a metal grid bottom for 1 h to allow accommodation. Then, the time until the mouse withdrew the paw due to the mechanical stimulus was analyzed (PWL). The PWL was obtained as the mean of six consecutive assessments at each time point (at least 10 s between repeated measurements of the same paw).

Thermal sensitivity (Hargreaves Test). PWL to thermal stimulation was assessed with an automated testing device consisting of a light ray which was directed against the plantar surface of a mouse's hind paw after placing the mouse on a 32°C warm transparent surface area (Plantar Aesthesiometer, Ugo Basile, Varese, Italy). Mice were kept in the test cages for 1 h to allow accommodation. Before the actual measurement took place with a measuring light ray (intensity 13), the stand by light ray (intensity 5) was positioned under the mouse's hind paw manually, and the measuring light ray was switched on. The time until the mouse withdrew the paw due to the thermal stimulus was analyzed (PWL). The cut off time was 20 s. The PWL was obtained as the mean of six consecutive assessments at each time point (at least 10 s between repeated measurements of the same paw).

Hot plate test. Animals were placed in a Plexiglas cylinder on a heated plate maintained at 52 ± 0.2°C (Ugo Basile), and the latency to jump or to shake/vibrate a hind paw was recorded. Each animal was tested only once, since repeated testing in this assay can lead to latency changes.[27] The cut off time was 30 s.

Acetone test. To assess cold allodynia, a drop of acetone was applied onto the plantar side of one hind paw. The time the mice spent lifting, shaking, or licking the acetone-treated paw was recorded during an observation period of 2 min starting right after acetone application.

Zymosan-induced paw inflammation, mechanical hyperalgesia. Hind paw inflammation was induced by subcutaneous injection of 20 µl of a 10 mg/ml zymosan A (Sigma–Aldrich, Munich, Germany) suspension in phosphate-buffered saline (0.1 M, pH 7.4) into the mid plantar region of the left hind paw.[28] Baseline mechanical sensitivity was determined before zymosan injection as described above (Dynamic Plantar Test). Mechanical hyperalgesia was analyzed as the mean of four consecutive assessments at 10 s intervals starting 1 h after zymosan A injection and then hourly up to 8 h. Mechanical hyperalgesia was further assessed at the time points 24 h and 48 h after zymosan A injection.

Formalin test. The formalin test was performed as described.[29] Mice were placed on a table top within a Plexiglas cylinder and were allowed to habituate for 30 min; 20 µl of a 5% formaldehyde solution (formalin) were injected subcutaneously into the dorsal surface of the left hind paw. The time spent licking the formalin-injected paw was recorded at 5 min intervals up to 45 min, starting right after formalin injection.

Assessment of endocannabinoid levels

Serum samples were analyzed for arachidonoyl ethanolamide (AEA) and 2-AG. The deuterated substances AEA-d8 and 2-AG-d5 were used as internal standards. Two cycles of ethyl acetate extraction were performed; 50 µl ethyl acetate was added to 50 µl of the serum sample, vortexed and centrifuged for 3 min at 20,238 xg. The organic phase was removed, and the extraction was repeated with 150 µl of ethyl acetate. The two organic fractions were combined and evaporated at a temperature of 45°C under a gentle stream of nitrogen. The residues were reconstituted in 50 µl of acetonitrile and centrifuged for 3 min at 20,238 xg. The liquid phase was transferred to glass vials, and 10 µl were injected into the LC-MS/MS system.

LC analysis was done under gradient conditions using a Luna C18 (2) column (150 mm L × 2 mm ID, 5 µm particle size, Phenomenex, Aschaffenburg, Germany). MS/MS analyses were performed on a 5500 Q-TRAP hybrid triple quadrupole mass spectrometer with a Turbo V source (Sciex, Darmstadt, Germany) in the negative ion mode. Precursor-to-product ion transitions of m/z $346.0 \rightarrow 259.0$ for AEA, m/z $354.0 \rightarrow 266.8$ for AEA-d8, m/z $377 \rightarrow 303.0$ for 2-AG, and m/z $382.1 \rightarrow 303.0$ for 2-AG-d5 were used for the multiple reaction monitoring with a dwell time of 40 ms. Concentrations of the calibration standards, quality controls, and samples were evaluated by MultiQuant-Software 3.0 (Sciex, Darmstadt, Germany).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from spinal cord tissue as described previously[30]; 200 ng of total RNA was used for the reverse transcription, which was performed with the Thermo Scientific Verso cDNA system (Thermo Fisher Scientific GmbH, Germany); 20 ng RNA equivalent were subjected to quantitative real-time PCR (qRT-PCR) in an Applied Biosystems Sequence Detection System AB7500 using SYBR Select Master Mix (Life Technologies, Germany) with SYBR Green fluorescence staining. Expression of PCR products were determined and normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA, which was detected in the same way. The following gene-specific primers were used:

CB1 receptor:
FW 5′-TCTCATAGAGTCTGGGGGCA-3′,
RV 5′-GAGGGAACCCCTTCGCATC-3′,
IL1beta:
FW 5′-CTGGTGTGTGACGTTCCCATTA-3′
RV 5′-CCGACAGCACGAGGCTTT-3′
TNFalpha:
FW 5′-GCTGAGCTCAAACCCTGGTA-3′
RV 5′-CGGACTCCGCAAAGTCTAAG-3′
c-fos:
FW 5′-ACCATGATGTTCTCGGGTTTCAA-3′
RV 5′-GCTGGTGGAGATGGCTGTCAC-3′
COX-2:
FW 5′-
AGACACTCAGGTAGACATGATCTACCCT-3′,
RV 5′-GGCACCAGACCAAAGACTTCC-3′
GAPDH:
FW 5′-CAATGTGTCCGTCGTGGATCT-3′
RV 5′-GTCCTCAGTGTAGCCCAAGATG-3′

The primers for fatty acid amid hydrolase (FAAH) were purchased from Life Technologies (Taqman Gene Expression Assay). The cycle number at which the fluorescence signals cross a defined threshold (Ct-value) is proportional to the number of RNA copies present at the start of the PCR. The threshold cycle number for the specific mRNA was standardized by subtracting the Ct-value of GAPDH from the Ct-value of the specific PCR product of the same sample. Relative mRNA quantities were determined by standard $2^{(-\Delta\Delta Ct)}$ calculation and expressed as fold-change of reference samples (mice fed ad libitum).

Cell incubation and Western Blot analysis

Human embryonic kidney cells (HEK293) (LGC Standards, Wesel, Germany) were cultured in Dulbecco's Modified Eagle Medium + Glutamax™ + 4,5 g/l Glucose (Gibco/Life Technologies, Carlsbad, USA) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin in an incubator at 37°C and 5% CO_2. For the experiments, cells were seeded at a density of 1×10^6 in cell culture dishes (10 cm diameter). After 24 h, the cell culture medium was replaced by cell culture starving medium without FCS; 48 h after seeding, the cells were incubated with AEA (10 μM) (dissolved in EtOH at 5 mg/ml) with and without addition of the cannabinoid receptor 1 antagonist AM251 (1 μM) (Tocris) and/or the cannabinoid receptor 2 antagonist A260 (Sigma) for 1 h. Controls were treated with solvent only. Then, the medium was discarded, cells washed with sterile PBS for two times, and then harvested using a rubber policeman. Cell lysis was performed in PhosphoSafe Extraction Buffer (Merck, Darmstadt, Germany) containing protease inhibitor (1 mM Pefabloc SC, Alexis Biochemicals, Lausen, Switzerland). To remove cellular debris, extracts were centrifuged at 16,800 × *g* for 15 min at 4°C. The protein containing supernatant was stored at −20°C until further analysis.

Proteins (30 μg) were separated electrophoretically by 10% SDS-PAGE and then transferred onto nitrocellulose membranes by wet blotting. To confirm equal loading, all blots were stained with Ponceau red solution. Membranes were blocked for 60 min at room temperature in Odyssey blocking reagent (LI-COR Biosciences, Bad Homburg, Germany) diluted 1:2 in 0.1 M PBS, pH 7.4. Then the blots were incubated overnight at 4°C with primary monoclonal antibody against pAMPKα and AMPKα (antigen: synthetic phosphopeptide corresponding to residues surrounding Thr172 or the amino-terminal sequence of human AMPKα, respectively, Cell Signaling #2535, #2603, Heidelberg, Germany, 1:250) in blocking buffer. After washing three times with 0.1 % Tween 20 in PBS, the blots were incubated for 60 min with an IRDye 700-conjugated secondary antibody (goat-anti-rabbit IRDyeTM 680; 1:5.000 in blocking buffer). After rinsing in 0.1 % Tween 20 in PBS, protein–antibody complexes were detected with the Odyssey Infrared Imaging System (LI-COR, Bad Homburg, Germany). Anti-GAPDH (mAB, antigen: purified rabbit muscle GAPDH (whole molecule), Thermo-Fisher Scientific AM4300, Germany, 1:1.200) was used as loading control. Densitometric analysis of the blots was performed with Quantity One software (Bio-Rad; München, Germany).

Data analysis

Statistical evaluation was done with Graph Pad Prism 5.0 for Windows. Data are presented as mean ± SEM. Data were tested for Gaussian distribution by the Kolmogorov-Smirnov test and were then either compared by univariate analysis of variance (ANOVA) with subsequent *t* tests employing a Bonferroni

α-correction for multiple comparisons or a Dunnet test or by student's *t* test. For analysis of inflammatory hyperalgesia in the zymosan A induced paw inflammation, paw withdrawal latencies in response to mechanical stimulation were expressed as the relative difference between the zymosan A-treated left and the untreated right hind paw calculated as: $\Delta PWL = (\text{left-right})/\text{right-} \times 100$ (mean ± SEM). The area under the PWL versus time curve (AUC) was calculated by employing the linear trapezoidal rule. For all tests, $P < 0.05$ was considered as statistically significant.

Results

Caloric restricted mice show a reduced nociceptive behavior in inflammatory models

Mice were fed either ad libitum (100%) or received a CR to 80% of their average daily food quantity for a period of 14 days. Their general well-being as well as the body weight were documented every other day. Mice of either group showed normal activity and roaming behavior. However, while six to eight-weeks-old mice gained about 6% body weight within two weeks, mice that underwent two weeks of CR with 80% of the daily average lost around 15% body weight.

Since intact motor function is an important prerequisite for a proper analysis of the nociceptive behavior, we performed the Hanging wire test. All animals were able to stay at the wire until the cut off time of 90 s indicating that motor function is not influenced by CR (not shown results). The nociceptive behavior was compared in four models of acute nociception based on thermal and mechanical noxious stimulation of the paw. C57BL/6 wild type mice on CR displayed less sensitivity to acute noxious cold and heat stimuli than mice fed normally indicating that the thermal acute nociceptive system is less sensitive after CR. In contrast, we did not find differences in the mechanical nociceptive threshold in both groups (Figure 1). Since activation of AMPK has been reported after CR for several times[14,15] and has also been associated with antinociceptive effects,[19,31,32] we further

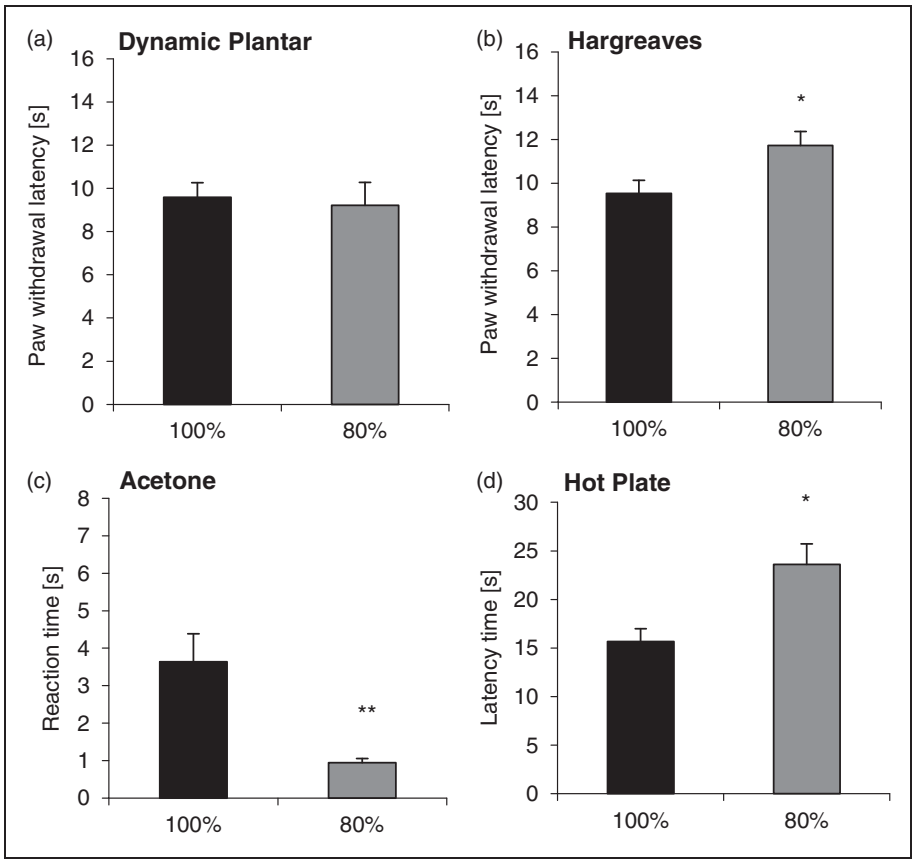

Figure 1. Acute nociception. C57BL/6 mice fed ad libitum (100%) and mice on caloric restriction (80%) ($n = 6$ mice/group) compared concerning (a) paw withdraw latency after a mechanical stimulus with a Dynamic Plantar Aesthesiometer, (b) paw withdraw latency after a heat stimulus with a light ray in a Hargreaves apparatus (Student's *t* test: $P = 0.0168$, $F = 1.282$), (c) latency time of reaction to a drop of acetone applied to a paw (Student's *t* test: $P = 0.0051$, $F = 37.92$), (d) latency time until reaction on a hot plate (Student's *t* test: $P = 0.0128$, $F = 2.581$). *$P < 0.05$, **$P < 0.01$, statistically significant difference.

assessed the impact of an AMPK deletion in these models. Interestingly, neither AMPKα2 knock-out mice nor wild type littermates on CR showed alterations in their response to acute noxious stimuli in comparison to mice fed normally indicating that the acute nociceptive system is not influences by CR in these mice (Supplementary Figure 1).

In addition, we investigated the nociceptive response in the two differently fed mouse groups in two models of inflammatory nociception, the formalin test and the zymosan-induced paw inflammation. The formalin test is characterized by two phases. Phase 1 lasts from time point 0 until 10 min and reflects an acute nociceptive response to the formalin injection into the paw. After a brief gap, phase 2 starts as a correlate for short-term inflammatory nociception and lasts approximately until 45 min after formalin injection. In the first phase, there were no differences between ad libitum fed and CR mice; however in the second phase, a drastic reduction of the nociceptive response in CR mice could be observed which is statistically significant (Figure 2(a)). The zymosan induced paw inflammation is characterized by longer lasting symptoms of inflammation, e.g. edema, redness, and hyperalgesia. After injection of zymosan A into the plantar surface of one hind paw, mice fed ad libitum showed a typical strong decrease in the paw withdrawal latencies in response to a mechanical stimulus indicating the development of mechanical hyperalgesia. This response was significantly alleviated in mice fed only 80% of their daily average as indicated by an increase in the area under the PWL versus time curve (AUC) diagram (Figure 2(b)).

AMPK activation is associated with a decrease of inflammatory nociception.[19] Therefore, we assumed that the kinase might be involved in the diet-induced reduction of inflammatory hyperalgesia. Since longer lasting effects appeared more important after long-term CR, we assessed the nociceptive response after CR in the zymosan model also in *AMPKα2* knock-out mice. Interestingly, and similar to results in the acute models, the significant antihyperalgesic effect of CR observed in C57BL/6 wild type mice was less pronounced in AMPKα2 wild type littermates. However, in the *AMPKα2* knock-out mice inhibition of inflammatory hyperalgesia was completely diminished (Figure 2(c) and (d)) indicating that AMPK might be involved in the decreased inflammatory nociception after CR.

Effect of CR on endocannabinoid levels and CB1 receptor expression

To further assess the molecular mechanisms which might induce antinociception after CR, we performed qRT-PCR analyses to evaluate levels of typical proinflammatory genes such as the cytokines IL-1β and TNFα as well as cyclooxygenase 2 (COX-2) and the typical spinal marker for neuronal activity, c-fos 48 h after zymosan injection into the hind paw. Interestingly, none of these genes showed differences in C57BL/6 mice with and without CR (Figure 3). Therefore, it was likely that other mechanisms are responsible for the observed reduced nociceptive behavior. Instead of inhibition of the proinflammatory response, it may be suggested that CR might activate endogenous inhibitory systems. Since endocannabinoids have already been linked to dietary responses as well as antinociception, we intended to investigate the impact of the endocannabinoid system on the decreased acute and inflammatory nociception after CR. On one hand, we assessed the amount of CB1 receptor in the spinal cord of differently fed mice using qRT-PCR and, on the other hand, serum AEA and 2-AG levels in the same mice using LC-MS/MS. Basal levels of CB1 receptor in the spinal cord (Figure 4) as well as endocannabinoid levels in the serum (Figure 5) were similar in mice fed 100% or 80% of their daily average. These results indicate that CR-induced differences in the response to acute noxious thermal stimuli are not mediated by endocannabinoids. However, 48 h after zymosan A injection, levels of CB1 receptor in the spinal cord (Figure 4) were significantly higher in mice that underwent CR compared to control mice. Endocannabinoid levels of AEA and 2-AG in the serum increased after zymosan injection but showed no differences between mice on 100% and 80% food, respectively. This observation corresponds well with no changes of the endocannabinoid hydrolyzing enzyme FAAH after CR (Figure 5) as assessed by qRT-PCR analyses. These results point to an increased sensitivity of the endogenous endocannabinoid system at the receptor level leading to the positive antinociceptive effect of CR in the zymosan A-induced paw inflammation. Endocannabinoids have already been associated with AMPK activation,[21] and therefore, an interplay of both systems might constitute a mechanism of CR-induced antinociception. To confirm that AMPK activation is involved in endocannabinoid signaling, we incubated HEK293 cells with AEA and analyzed phosphorylation of AMPK by Western Blot. The results showed an increase in pAMPK which could be inhibited by the CB1 receptor antagonist AM251 but not the CB2 receptor antagonist AM630 thus indicating AMPK activation by endocannabinoids via the CB1 receptor (Figure 6).

Discussion

This study was designed to investigate the effects of prolonged moderate CR on acute and inflammatory nociception in mice. Furthermore, we intended to elucidate molecular mechanisms influenced by the dietary changes.

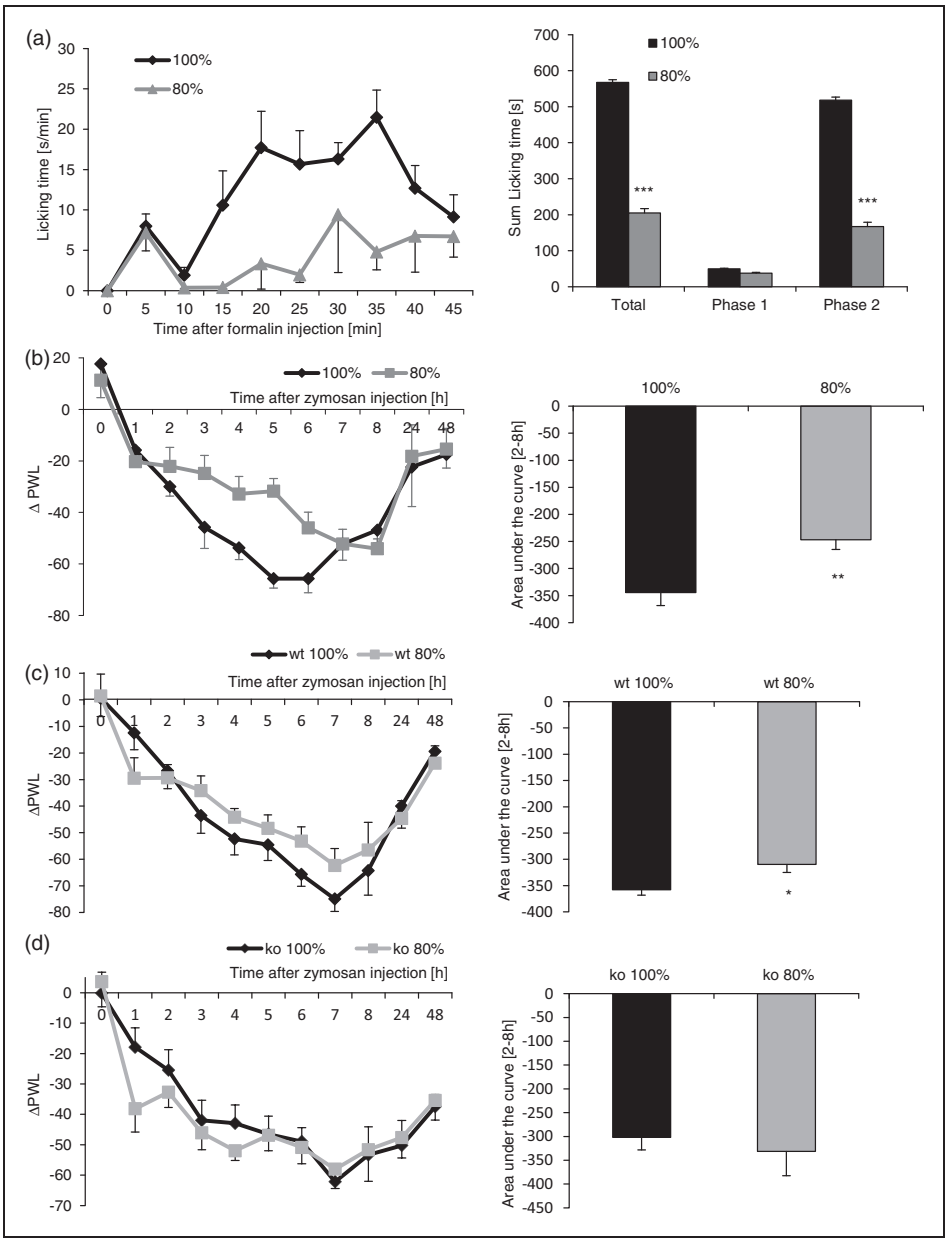

Figure 2. Inflammatory nociception in C57BL/6 wild type mice with and without CR. (a) Left: Time course of the nociceptive behavior of C57BL/6 wild type mice fed 100% (♦) or 80% of their average daily food quantity (▲) after injection of 5% formalin (20 µl) solution into a hind paw (repeated measures ANOVA: $P = 0.0005$, $F = 25.97$). Right: Comparison of the total licking and the licking times in phase 1 and phase 2 between wild type mice fed 100% and wild type mice on caloric restriction fed 80% of their average daily food quantity (Student's *t* test: total: $P = 0.0005$, $F = 2.627$, phase 2: $P = 0.0009$, $F = 2.027$) ($n = 6$ mice/group). (b) Left: Timecourse of mechanical hyperalgesia in C57BL/6 wild type mice fed 100% (♦) or 80% of their average daily food quantity (■) after injection of 10 mg/ml (20 µl) zymosan A into a hind paw. The diagram shows the delta paw withdrawal latencies (ΔPWL) in response to mechanical stimulation as assessed with a Dynamic Plantar Aesthesiometer (repeated measures ANOVA: $P = 0.05$, $F = 4.96$). Right: Comparison of the area under the PWL versus time curve (AUC) between wild type mice fed 100% and wild type mice on caloric restriction fed 80% of their average daily food quantity 2 to 8 h after zymosan A injection (Student's *t* test: $P = 0.0087$, $F = 1.803$) ($n = 6$ mice/group). (c) Left: Timecourse of mechanical hyperalgesia in AMPKα2 wild type littermates fed 100% (♦) or 80% of their average daily food quantity (■) after injection of 10 mg/ml (20 µl) zymosan A into a hind paw (repeated measures ANOVA: $P = 0.0917$, $F = 3.28$). The diagram shows the delta paw withdrawal latencies (ΔPWL) in response to mechanical stimulation. Right: Comparison of the AUC between mice fed 100% and mice on caloric restriction fed 80% of their average daily food quantity 2 to 8 h after zymosan A injection (Student's *t* test: $P = 0.0352$, $F = 2.196$) ($n = 8$ mice/group). (d) Left: Timecourse of mechanical hyperalgesia in AMPKα2 knock-out mice fed 100% (♦) or 80% of their average daily food quantity (■) after injection of 10 mg/ml (20 µl) zymosan A into a hind paw. The diagram shows the delta paw withdrawal latencies (ΔPWL) in response to mechanical stimulation. Right: Comparison of the AUC between AMPKα2 knock-out mice fed 100% or 80% of their average daily food quantity 2 to 8 h after zymosan A injection ($n = 8$ mice/group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, statistically significant difference.

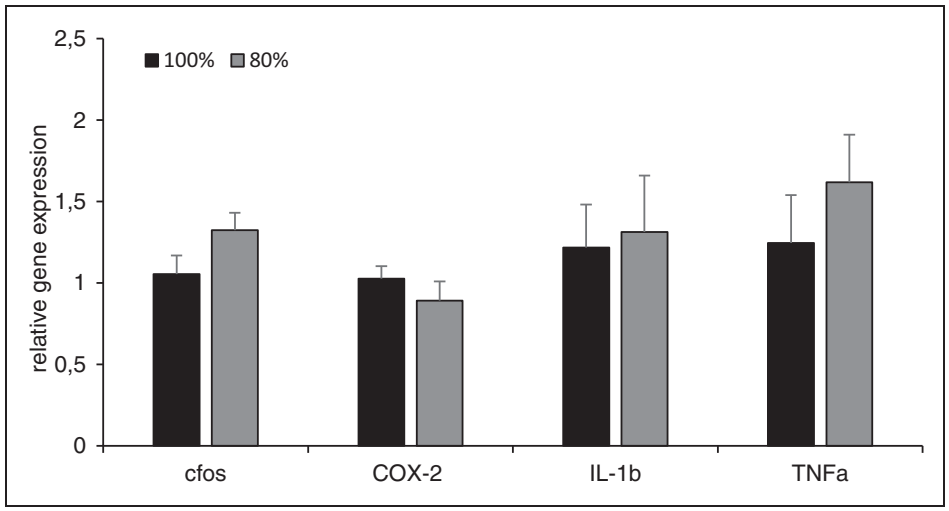

Figure 3. Regulation of inflammatory genes in the zymosan A-induced paw inflammation. Relative mRNA expression of c-fos, COX-2, TNFα, and IL1β in the spinal cord of mice with and without CR 48 h after zymosan A injection into the paw as assessed by qRT-PCR ($n = 9$ mice in the 100% fed group, $n = 12$ mice in the 80% fed group).

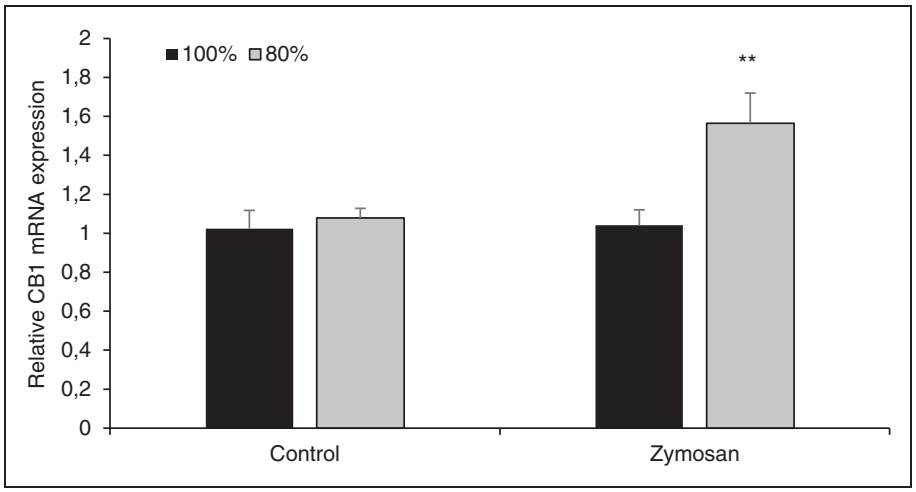

Figure 4. Effect of caloric restriction on CB1 receptor expression in spinal cord of mice with and without nociceptive stimulation. CB1 receptor mRNA expression in the spinal cord of mice fed 100% and mice on caloric restriction fed 80% of their average daily food quantity without nociceptive stimulation and 48 h after zymosan A injection into the hind paw as assessed by qRT-PCR (Student's *t* test: 100% zymosan vs. 80% zymosan, $P = 0.0011$, $F = 3.635$) ($n = 6$/group). For better comparison, mRNA levels of mice fed 100% have been set as 1. $^{**}P < 0.01$, statistically significant difference.

Our results revealed that thermal acute nociception is inhibited in calorie-restricted C57BL/6 wild type mice which correspond with results from already published studies in the context of CR-induced antinociception.[4–6,16] Mechanical nociception was not affected by the diet which is also in line with already published results.[33] Further experiments showed a strong inhibition of inflammatory nociception due to calorie restriction in two models of inflammation. Since changes in food intake have an impact on a number of different signaling pathways, we assessed several different potentially modulated candidates. In our experiments, expression of proinflammatory and pronociceptive marker genes was not altered by CR indicating that they are not involved in the antinociceptive effects. These observations are in line with contradictory results concerning influence of CR on inflammatory mediators. On the one hand, CR-induced increases in cytokine levels were suggested to be due to social stress by the diet[5] and on the other hand decreases in inflammatory mediators might contribute to anti-inflammation.[34,35] The differential responses might also depend on the extent and duration of CR.

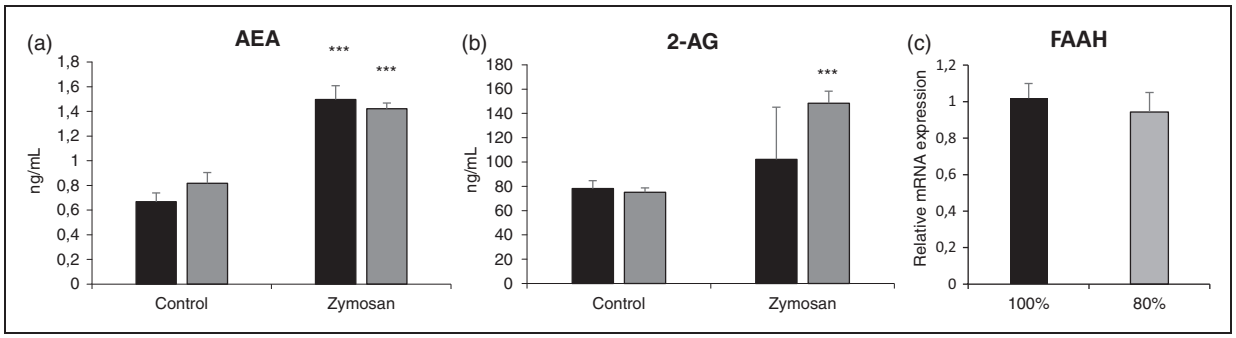

Figure 5. Effect of caloric restriction on serum levels of AEA and 2-AG in mice with and without nociceptive stimulation. Serum AEA (a) and 2-AG (b) levels of mice fed 100% (black columns) and mice on caloric restriction fed 80% of their average daily food quantity (gray columns) without nociceptive stimulation and 48 h after zymosan A injection into the hind paw as assessed by LC-MS/MS (Student's *t* test: AEA control 100% vs. zymosan 100%, $P = 0.0002$, $F = 2.499$; control 80% vs. zymosan 80%, $P = 0.0006$, $F = 3.606$; 2-AG control 80% vs. zymosan 80%, $P = 0.0002$, $F = 7.86$). Spinal FAAH mRNA (c) of mice fed 100% or 80% of their average daily food quantity, respectively, 48 h after zymosan A injection ($n = 6$/group). $^{***}P < 0.001$ statistically significant difference between control and zymosan A-treated group.

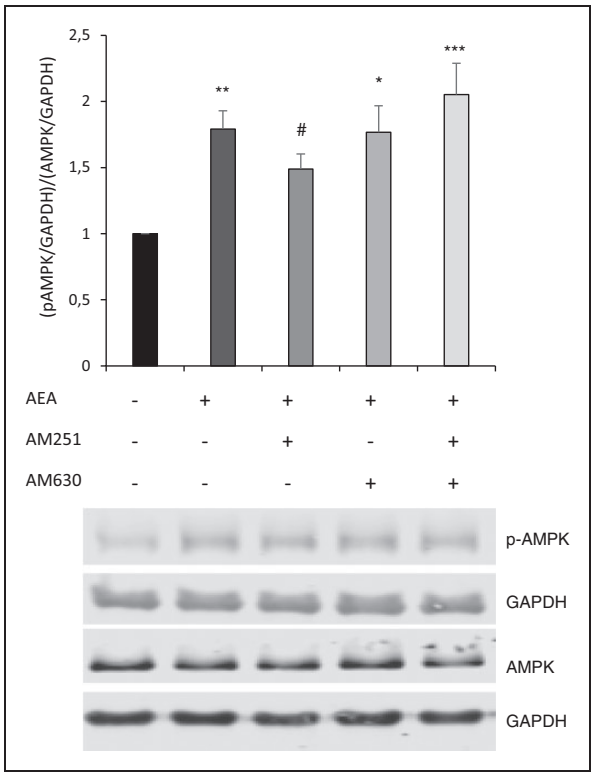

Figure 6. Activation of AMPK after endocannabinoid treatment of HEK293 cells. Western Blot analysis of pAMPKα vs. AMPKα levels in HEK293 cell after treatment with 10 μM arachidonoyl ethanolamide (AEA) with and without addition of the CB1R antagonist AM251 (1 μM) or the CB2R antagonist AM630 (1 μM) or vehicle for 1 h, respectively. GAPDH served as loading control (one-way ANOVA: $P = 0.0015$, $F = 5.552$; Student's *t* test: AEA vs. AEA/AM251, $P = 0.0440$, $F = 1.648$). The diagram shows the densitometric analysis of all experiments ($n = 8$), the blots one representative result. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ statistically significant difference in comparison to vehicle, $^{\#}P < 0.05$ statistically significant difference in comparison to AEA.

Activation of the AMPK has already been described after CR.[14,15] A role of AMPK in CR is further supported by the fact that CR-mimetics activate AMPK, and that AMPK activation, similar to CR, is involved in longevity and the prevention of age-related diseases.[36] Furthermore, pharmacological activation of AMPK has been associated with a decrease in nociception in models of inflammatory and neuropathic pain[19,31,32] which could be explained by decreased activation of MAP-kinases or changes in the cells translational machineries, respectively. These data indicate the potential role of AMPK in CR-induced antinociception. Our data showed that, in contrast to C57BL/6 mice, reduction of caloric intake to 80% neither affected acute nociception of AMPKα2 wild type nor knock-out littermates which might be due to strain differences by backcrossing and does not allow a clear statement on the role of AMPK after CR in acute nociception. However, in inflammatory models, our results revealed that CR-induced antinociceptive effects observed in both C57BL/6 mice and AMPK wild type mice do not occur in AMPKa2 knock-out mice indicating that diet-induced AMPK-activation might be at least partially involved in the molecular mechanisms leading to antihyperalgesia after CR. As a further potential mediator of CR-induced antinociception, we investigated whether or not endogenous cannabinoid signaling is modulated by CR. Increases in endocannabinoid levels have been reported in mice and men after short-term fasting periods.[10,17,37] However, longer term moderate restriction diet (40%–60% for 12 days) decreased endocannabinoid levels of 2-AG.[37] The observed increase in endocannabinoids might be due to reduced leptin levels after CR.[38,39] A decrease in leptin as well as leptin insensitivity have just recently been associated with analgesic effects in combination with an increase in 2-AG.[40] In our experiments,

levels of AEA and 2-AG were not influenced by 14 days of CR to 80% of the daily average with and without inflammatory stimulation. However, CB1 receptor expression increased significantly in response to combined CR and inflammation, indicating that, despite a lack of changes in endocannabinoid levels, activity of the endocannabinoid system is enhanced by an increase in CB1 receptors and might contribute to the mechanisms of CR-induced inhibition of inflammatory hyperalgesia. In contrast, the decreased acute thermal nociceptive responses in caloric restricted mice is independent from endocannabinoids since neither spinal CB1 receptor expression nor serum AEA levels were regulated by CR only. These data indicate that CR effects differ between inflammatory and non-inflammatory conditions possibly due to regulation of different neurotransmitter systems such as opioid receptors or adrenocortical hormones.[6,41]

Since endocannabinoid-dependent changes in AMPK activation have already been shown in CB1 receptor knock-out mice[42] and in cell culture,[43] we hypothesize that CR-induced antinociception might be mediated by AMPK activation in response to increased activity of the endocannabinoid system which is supported by AMPK activation by AEA treatment. AMPK might then modulate similar genes as already shown in studies which investigated the effects of pharmacological AMPK activation in models of nociception.[19,31,32] Reduced activation of mTOR or MAPKs could contribute to antinociceptive responses in this study.

Taken together, our data reveal that CR influences acute and inflammatory nociceptive responses. In zymosan-induced paw inflammation, the CR-induced antihyperalgesia might comprise activation of the endogenous endocannabinoid system by CR-induced CB1 receptor upregulation and AMPK activation.

Author contributions

TSKH planned the experiments and performed animal, biochemical, and molecular biological analyses and analyzed data, CVM was involved in the preparation of tissue samples and qRT-PCR analysis, MCW was involved in animal treatment an preparation of tissue samples, JS participated in statistical analysis of the results, MM performed the experiments required for the revision of the manuscript, YS performed preparation of the samples and LC-MS/MS analyses, NF established the LC-MS/MS analyses, GG participated in the design of the study and editing of the manuscript. EN conceived and designed the study, coordinated subprojects, analyzed data, and wrote the manuscript. All authors have read and approved the final manuscript.

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Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: None of the authors has a conflict of interest.

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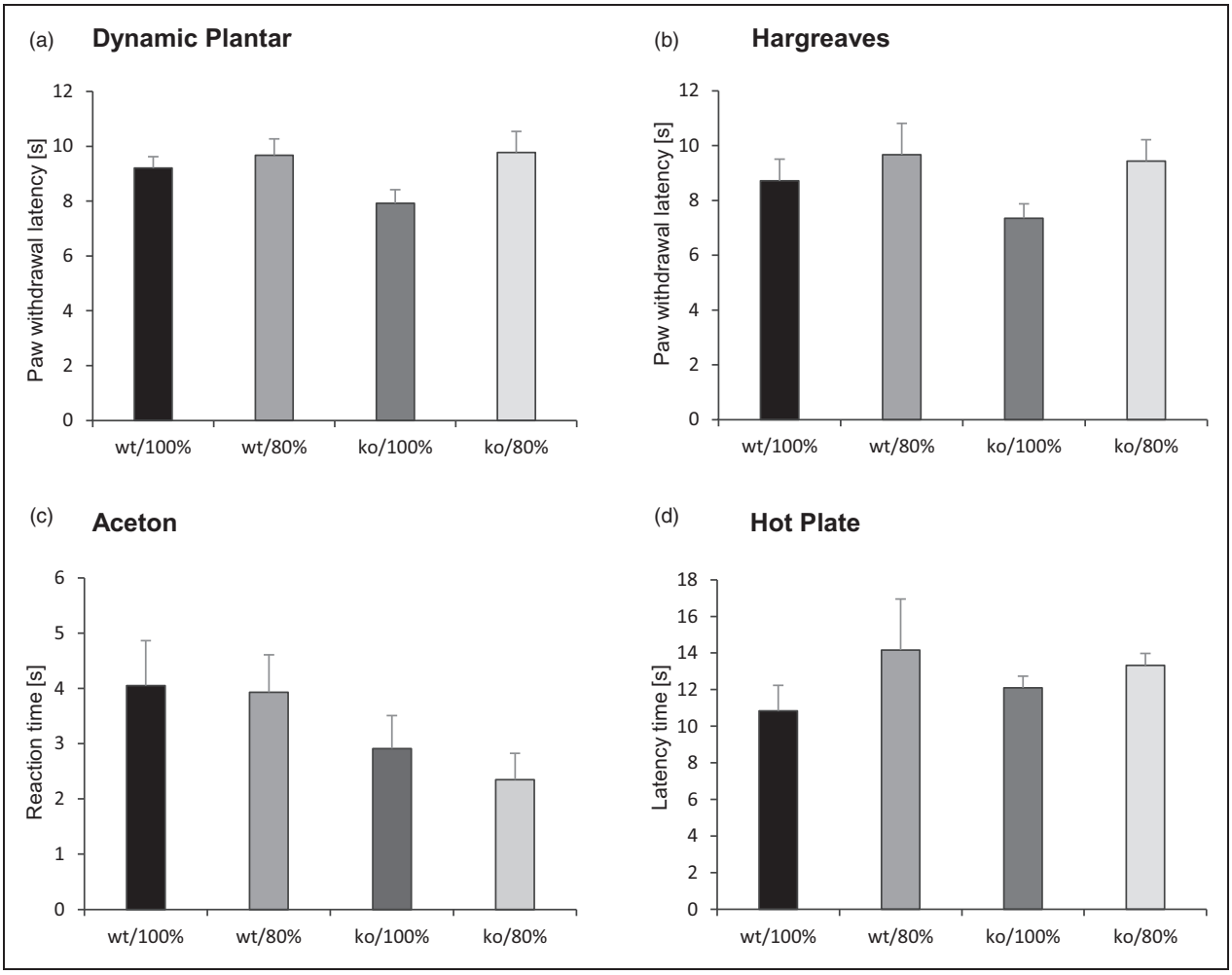
Supplementary Marterial



Suppl. Figure 1. Acute nociception in AMPKa mice.