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ENP11, a potential CB1R antagonist, induces anorexia in rats

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ABSTRACT

Over the past decade, pharmacological manipulation of cannabinoid 1 receptor (CB1R) has become an interesting approach for the management of food ingestion disorders, among other physiological functions. Searching for new substances with similar desirable effects, but fewer side-effects we have synthesized a SR141716A (a cannabinoid receptor inverse agonist also called Rimonabant) analog, 1-(2,4-Difluorophenyl)-4-methyl-N-(1-piperidinyl)-5-[4-(trifluoromethyl)phenyl]-1H-pyrazole-3-carboxamide, ENP11, that so far, as we have previously shown, has induced changes in glucose availability, *i.e.* hypoglycemia, in rats. In this study we tested the effects, if any, of ENP11 (0.5, 1.0, and 3.0 mg/kg) in food ingestion, core temperature, pain perception and motor control in adult Wistar rats.

Results showed that ENP11 reduced food ingestion during the first hour immediately after administration. Likewise, ENP11 (1.0 mg/kg) blocked anandamide (AEA)-induced hyperphagia during the first 4 h of the dark phase of the light–dark cycle, and it also blocked AEA-induced hypothermia. However, none of the ENP11 doses used affected pain perception or motor control.

We believe that ENP11 is a potential useful CB1R antagonist that reduces food ingestion and regulates core temperature.

1. Introduction

The incidence of obesity and associated metabolic disorders has become a health problem worldwide (World Health Organization, WHO 2015). Due to the fact that extensive scientific literature has supported the endocannabinoid system (eCBs) as a food ingestion modulator, the phyto and synthetic cannabinoids seem to be useful to affect such a function (Koch, 2001; Martínez-González et al., 2004; Méndez-Díaz et al., 2012; Merroun et al., 2009; Pérez-Morales et al., 2012; Soria-Gómez et al., 2010). In this context, it is now known that CB1R antagonists reduce food intake thereby promoting weight loss. Moreover, CB1R antagonists improve the metabolic profile in dogs, rodents and humans (Bennetzen et al., 2008; Bergholm et al., 2013; Cota et al., 2009; Van Gaal et al., 2008, Mølhøj et al., 2010; Randall et al., 2010; Richey et al., 2009; Van Gaal et al., 2005; Verty et al., 2009).

CB1R is widely expressed throughout the central nervous system in presynaptic GABAergic and glutamatergic terminals (Huang et al., 2001; Katona et al., 1999; Marsicano and Lutz, 1999). It is a protein Gi coupled receptor that promotes potassium channel opening while blocking N/P/Q-type calcium channels, thus reducing neuronal excitability and neurotransmitter release (Twitchell et al., 1997). Although the mechanism by which CB1R activation induces food intake is not quite clear, we believe it is part of a negative feedback loop that regulates neurotransmitter release, thereby modulating various CNS circuits, including those involved in food ingestion and energy expenditure such as the hypothalamic and reward systems. We recently synthesized a CB1R high affinity, SR141716A analog, to wit: 1-(2,4-Difluorophenyl)-4-methyl-N-(1-piperidinyl)-5-[4-(trifluoromethyl)phenyl]-1H-pyrazole-3-carboxamide (ENP11). We have shown that ENP11 causes hypoglycemic effects in experimentally induced diabetic rats. ENP11-induced food ingestion reduction was an expected effect based on the estimation made by the Prediction of Activity Spectra for Substances (PASS®) program (Hernández-Vázquez et al., 2013).

1.1. Present study

In this study we have tested the effects of ENP11 on food ingestion, pain perception, core temperature, and motor control, seeking to

Abbreviations: ENP11, 1-(2,4-Difluorophenyl)-4-methyl-N-(1-piperidinyl)-5-[4-(trifluoromethyl)phenyl]-1H-pyrazole-3-carboxamide; CB1R, cannabinoid 1 receptor; AEA, anandamide; eCBs, endocannabinoid system; DMSO, dimethyl sulfoxide.

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describe a potential influence of ENP11 on these variables. To further support its properties to antagonize endocannabinoid activity, we tested its efficacy to inhibit anandamide (AEA)-induced effects on these variables and compare it to AM251 and Rimonabant efficacy.

2. Material and methods

2.1. Animals

Adult male Wistar rats (250 g) were used. Rats remained housed in standard Plexiglas cages (42 × 25.5 × 20 cm) with sawdust bedding and maintained on a controlled 12/12 h light/dark cycle (lights on 20:00 h), in climate controlled rooms (21 ± 1 °C and 52% humidity). Water and food (Rat Chow, Purina) were provided *ad libitum*. The rats were allowed one week of habituation prior to experimental manipulation. All rats were used in one experiment only. Every effort was made to minimize the number of animals used and their potential suffering. All experiments were carried out during the dark phase of the photoperiod. The management and animal care adhered strictly to the provisions of the Official Mexican Regulation on "Technical specifications for the production, care and use of laboratory animals" (NOM-062-ZOO-1999). Additionally, our protocol was approved by the Research and Ethics Committee of the Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM).

2.2. Food ingestion

The election of dose used to evaluate the effect of ENP11 on food ingestion is based on the fact that 1 mg/kg SR141716A reduces food ingestion (Gómez et al., 2002; Dore et al., 2014), hence, we decided to use an equimolar dose of ENP11 that is 1 mg/kg, as well as a lower dose, 0.5 mg/kg and a higher dose, 3 mg/kg.

Rats were randomly assigned to each one of the following groups (n = 8–11): DMSO (300 μl), ENP11 (0.5, 1.0 or 3.0 mg/kg), SR141716A (1 mg/kg), AM251 (1.2 mg/kg), AEA (1 mg/kg), AEA (1 mg/kg)/ENP11 (1 mg/kg); AEA (1 mg/kg)/AM251 (1.2 mg/kg), AEA (1 mg/kg)/SR141716A (1 mg/kg). Rat body weight was measured immediately before treatment. Once rats received the treatment for the group they belonged to, the amount of food consumption was quantified 1 h later, and every hour, during the first 4 h of the dark phase of the cycle, and 24 h later. The amount of food consumed is reported as an index of the amount of food eaten (g) divided by the rat's body weight (g) × 100. Data are expressed as mean ± SEM. Data analyses were carried out every hour during the first 4 h, for the total 4 h, and for the total of 24 h, by means of a repeated-measures ANOVA test ($p < 0.05$).

2.3. Core temperature

Rats were randomly assigned to one of the following treatment groups (n = 5–8): DMSO (300 μl), AEA (1 mg/kg), ENP11 (1 mg/kg), SR141716A (1 mg/kg), AM251 (1.2 mg/kg), AEA (1 mg/kg)/ENP11 (1 mg/kg), AEA (1 mg/kg)/SR141716A (1 mg/kg), AEA (1 mg/kg)/AM251 (1.2 mg/kg). Core temperature was measured using a rectal thermometer with a digital display. Once rats were weighed, they received the corresponding treatment and returned to their home cage. Core temperature was measured 30, 60 and 240 min after administration. Data are expressed as mean ± SEM and were analyzed using a one-way repeated-measures ANOVA test followed by post-hoc Tukey test ($p < 0.05$).

2.4. Pain perception

Rats were randomly assigned to one of the following treatment groups (n = 7 each group): DMSO, AEA (1 mg/kg), ENP11 (1 mg/kg), SR 141716A (1 mg/kg), AM251 (1.2 mg/kg), AEA (1 mg/kg)/ENP11 (1 mg/kg), AEA (1 mg/kg)/SR141716A (1 mg/kg), AEA (1 mg/kg)/AM251 (1.2 mg/kg). The pain perception test was performed using a Hot Plate device (Socrel, model DS37). The stainless steel plate (25 × 25 cm) was maintained at 55 °C ± 1 °C. Rats were placed on the plate and maintained within the plate area by means of a Plexiglas cylinder (20 cm diameter and 20 cm height). The time elapsed from the moment the rat was placed on the plate to the time the rat showed signs of discomfort such as licking its hind paw or jumping to the highest border of the cylinder was recorded (s) and was considered the reaction time (latency). Rats received their treatment 30 min before the test. One additional assessment was performed 60 min after injection. The hot plate was cleaned thoroughly with a 5% chlorine solution after each trial. Data are expressed as mean ± SEM of latency (s) and were analyzed using a one-way ANOVA test followed by post-hoc Tukey test ($p < 0.05$).

2.5. Motor control

Rats were randomly assigned to one of the following treatment groups (n = 5–9): DMSO, AEA (1.0 mg/kg), ENP11 (0.5, 1.0, and 3.0 mg/kg), SR 141716A (1.0 mg/kg), AM251 (1.2 mg/kg), to evaluate motor control. We used a Rota-Rod (Ugo Basile Rota-Rod for rats 7750 Ugo Basile North America, PA, CA), the apparatus consists of a set of four drums, 50 cm high, on which 4 subjects are positioned simultaneously. These drums are separated by opaque disks to prevent the animals from being distracted. The speed of the drum's rotation increases steadily. Each drum has its own digital timer and display. The time elapsed from the time the rat is placed on the drum to the time the rat falls on a switch that shuts down a timer, is quantified.

The assay consists of 2 phases: the first phase is training, and it consists of placing the animals on the drum and turning on rotation 5 times, 1 min each time. Once the training phase is completed the animals are returned to their home cages allowing them to rest for 30 min. After this rest period the test begins, for which the animals are again placed on the Rota-Rod 5 more times, 1 min each time, while the drum's rotation increases steadily. In all trials, we quantified the time it took for the animals to fall from the drum (latency to fall). The Rota-Rod apparatus was cleaned thoroughly with a 5% chlorine solution after each trial. Data are expressed as mean ± SEM of time (s), and were analyzed using a one-way ANOVA test followed by post-hoc Tukey test ($p < 0.05$).

2.6. Drugs

AEA, SR141716A and DMSO were purchased from Sigma Aldrich Inc., MO, USA. AM251 was obtained from Tocris Bioscience MO, USA. AEA, Rimonabant, and AM251 were prepared in 100% DMSO. Drugs were injected intraperitoneally. Regular food used was Purina Lab Chow. ENP11 synthesis is described below.

2.7. ENP synthesis

The synthesis of ENP11 was published elsewhere (Hernández-Vázquez et al., 2013). Briefly, the enolate of substituted 4-trifluoromethyl propiophenone was obtained in methylcyclohexane by treatment with Lithium bis(trimethylsilyl)amide and was then treated with diethyl oxalate to obtain the tricarbonylic lithium salt in 70% yield. The next step was the cyclocondensation of 2,4-difluorophenylhydrazine and the previously obtained tricarbonylic compound in a sulfuric acid–ethanol solution, which afforded the ethyl pyrazole-5-carboxylate. The ester was converted to the corresponding carboxylic acid by treatment with potassium hydroxide at 50 °C, resulting in a yield of 59% for two steps. Finally, the target product was achieved with 81% yield by formation of the acyl chloride derivative from the pyrazole 5-carboxylic acid and the subsequent reaction with 1-aminopiperidine and N,N-Diisopropylethylamine in chloroform.

3. Results

The analysis per hour of ENP11 (0.5, 1.0 and 3.0 mg/kg) is depicted in Fig. 1a [$F(3,37) = 8.155$; $p = 0.0003$]. Tukey's analysis shows that the ENP11 at doses tested does not have significant effect on the amount of food ingested 1 h after its systemic administration ($p > 0.4$ *vs.* DMSO). During the 2nd h, ENP11 (1.0 and 3.0 mg/kg) decreases the amount of food ingested significantly compared to DMSO ($p = 0.049$ and $p = 0.048$ respectively). All ENP11 doses tested reduced the quantity of food ingested compared to DSMO 3 h after administration (ENP11 0.5 mg/kg $p = 0.006$; ENP11 1.0 mg/kg $p = 0.0001$ and ENP11 3.0 mg/kg $p < 0.0001$). ENP11 (1.0 and 3.0 mg/kg), significantly reduced food ingestion as compared to DMSO, 4 h after administration (ENP11 1.0 mg/kg $p = 0.028$ and ENP11 3.0 mg/kg $p < 0.0001$). 1 way ANOVA was used to analyze the 24 h effect of ENP11 [$F(3,37) = 4.680$, $p = 0.0072$]. Tukey's analysis shows that the higher ENP11 dose (3.0 mg/kg) tested decreased the total amount of food for the following 24 h ($p = 0.0038$) (insert 1a).

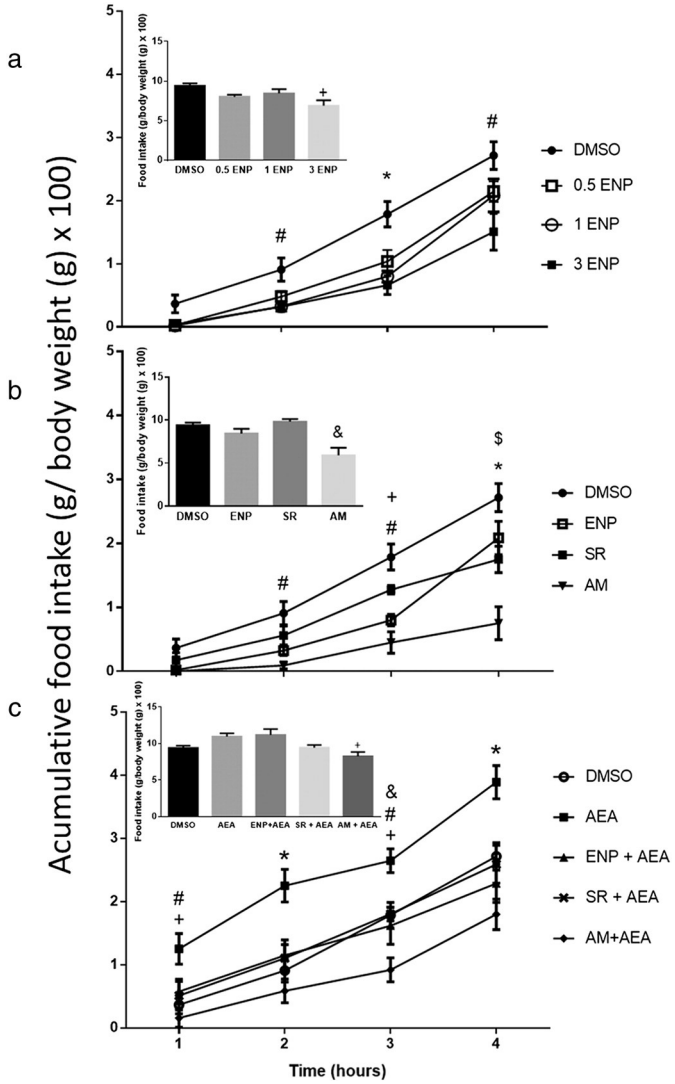

Fig. 1. Food intake. Graphs show the accumulative amount of food intake (g/body weight (g) × 100) during the first 4 h after the treatment and the inset shows the total amount of food intake (g/body weight (g) × 100) 24 h after the administration. a) Illustrates the effect of 0.5, 1.0 and 3.0 mg/kg of ENP 11. Two way ANOVA followed by Tukey, $^{*}p < 0.05$ DMSO *vs.* all doses of ENP 11; $^{\#}p < 0.05$ DMSO *vs.* 1.0 and 3.0 mg/kg of ENP 11; $^{+}p < 0.05$ DMSO *vs.* 3.0 mg/kg of ENP 11. b) Shows the effect of ENP 11 (1.0 mg/kg), SR141716A (1.0 mg/kg) and AM251 (1.2 mg/kg). Two way ANOVA followed by Tukey, $^{*}p < 0.05$ DMSO *vs.* ENP11, SR1417161A and AM251; $^{\#}p < 0.05$ DMSO *vs.* ENP11 and AM251; $^{\&}p < 0.05$ DMSO *vs.* AM251; $^{+}p < 0.05$ AM251 *vs.* SR141716A; $^{\$}p < 0.05$ AM *vs.* ENP and SR141716A. And c) shows the orexigenic effect of AEA (1 mg/kg), as well as antagonism of ENP11 (1 mg/kg), SR141716A (1 mg/kg), and AM251 (1.2 mg/kg). Two way ANOVA followed by Tukey, $^{*}p < 0.05$ AEA *vs.* all treatments; $^{\#}p < 0.05$ DMSO *vs.* AEA: $^{+}p < 0.05$ AEA *vs.* AM; $^{\&}p < 0.05$ AEA *vs.* ENP.

Regarding the effect of all CB1R antagonists Fig. 1b depicts the effect of ENP11 (1.0 mg/kg); SR141716A (1.0 mg/kg) and AM251 (1.2 mg/kg) on food ingestion [$F(3,35) = 13.40$; $p < 0.0001$]. Tukey's analysis revealed that none of the treatments affected significantly the amount of food ingested during the 1st h after administration ($p > 0.4$). However, during the 2nd h both AM251 ($p = 0.0038$) and ENP11 ($p = 0.0442$) reduced the amount of food ingested as compared to DMSO. The total amount of food ingested 3 h after ENP11 or AM251 administration was reduced as compared to DMSO ($p < 0.0001$ for both treatments). SR141716A does not affect food ingestion compared to DMSO group and therefore exhibits significant differences from AM251 effects ($p = 0.0041$). At the end of the 4th h post administration, all treatments reduced significantly the total amount of food ingested as compared to DMSO (ENP11 $p = 0.0252$; AM251 $p < 0.0001$ and SR141716A $p = 0.0001$). Both ENP11 and SR141716A treatments are significantly different from AM251 treatment ($p < 0.0001$ and $p = 0.0003$ respectively). The inset in Fig. 1b shows the 1 way ANOVA by 24 h [$F(3,34) = 9.677$; $p < 0.0001$]. Tukey's analysis shows that AM251 continues decreasing the amount of food ingested 24 h after administration ($p = 0.0003$ *vs.* DMSO) (inset 1b).

Two way ANOVA by h [$F(4,44) = 9.677$; $p < 0.0001$] reveals differences by AEA treatment. After Tukey's analysis, Fig. 1c shows the significant AEA-induced increase in food ingestion 1 h after its administration compared to DMSO ($p < 0.0384$), this effect was prevented by AM251 ($p = 0.0121$ *vs.* AEA). During the 2nd h all antagonists prevented AEA-induced increase in food ingestion (ENP11 $p = 0.046$; SR141716A $p = 0.0051$ or AM251 $p < 0.0001$ *vs.* AEA). During the 3rd h ENP11 ($p = 0.0100$) and AM251 ($p < 0.0001$) prevented AEA-induced increase in food ingestion. The analysis of the 4th h shows that ENP11 ($p < 0.0001$ *vs.* AEA), SR141716A ($p = 0.0010$ *vs.* AEA) or AM251 ($p < 0.0001$ *vs.* AEA) continues antagonizing the AEA effect. Tukey's analysis shows that AM251 remains antagonizing AEA-induced increase in food ingestion ($p = 0.0179$ *vs.* AEA) (inset 1c).

The effect of ENP11 (1.0 mg/kg), SR141716A (1.0 mg/kg) and AM251 (1.2 mg/kg) on core temperature 30, 60 or 240 min after administration was analyzed using 2 way RM ANOVA [$F(3,24) = 1.566$, $p = 0.2235$]. After Tukey's analysis we observed that no antagonist significantly affected the core temperature ($p > 0.05$ *vs.* DMSO) (Fig. 2a). Two way RM ANOVA was used to analyze AEA and AEA + antagonist treatments on core temperature 30, 60 or 240 min after administration [$F(4,38) = 3.090$; $p = 0.0090$]. Fig. 2b depicts the decrease in core temperature induced by AEA on core temperature 30 ($p = 0.0215$ *vs.* DMSO) and 60 ($p = 0.0018$ *vs.* DMSO) min after administration. This effect was blocked by ENP11 (1 mg/kg), SR141716A (1 mg/kg) and AM251 (1.2 mg/kg) at both times evaluated: 30 and 60 min ($p > 0.05$ *vs.* DMSO).

The effect of ENP11 (1.0 mg/kg), SR141716A (1.0 mg/kg) or AM251 (1.2 mg/kg) on pain perception was analyzed by two way ANOVA [$F(4,30) = 0.6348$, $p = 0.6416$] (Fig. 3a). Tukey's analysis shows that AEA (1.0 mg/kg) or any antagonist combination treatment affects pain perception ($p > 0.05$) (Fig. 3b).

Fig. 4 shows that none of the treatments used affected motor control tested in the Rota-Rod [$F(6,43) = 0.4618$, $p = 0.8326$].

4. Discussion

In the present study, we have described the pharmacological effect on food ingestion, core temperature, pain perception and motor control, of a recently synthesized CB1R antagonist, ENP11, a SR141716A related derivative, in rats. As expected ENP11 reduced food ingestion. ENP11 (1 mg/kg) effects on food ingestion were comparable to those induced

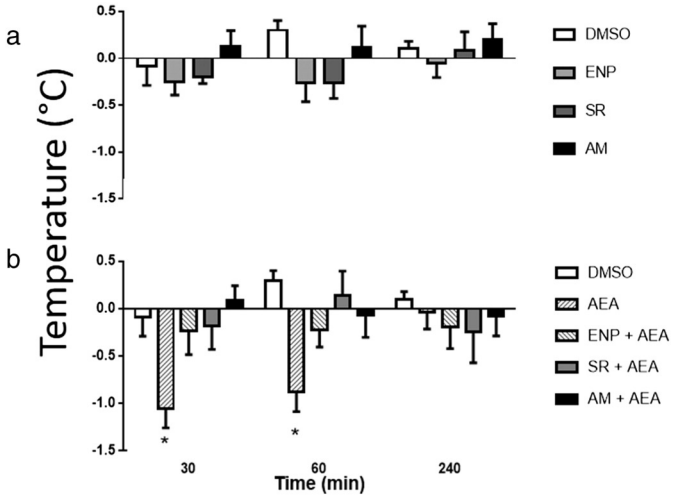

Fig. 2. Effect of ENP 11 on core temperature. Graph a) shows changes in core temperature (°C) 30, 60 and 240 min after administration of vehicle and antagonists ENP 11 (1 mg/kg), SR141716A (1 mg/kg), and AM251 (1.2 mg/kg); b) shows the hypothermic effect of AEA (1 mg/kg), as well as the antagonistic effect of ENP 11, SR141716A, and AM251 at the same doses used. *$p < 0.05$ AEA *vs.* all treatments.

by SR141716A or AM251 at equimolar dose used. ENP11 was unable to modify pain perception and motor control. Likewise, ENP11 prevented anandamide's effects on food ingestion and core temperature.

Although none of the antagonists administered had an effect on food ingestion 1 h post administration, in the 2nd h the expected effect of ENP11 appeared, that is similar to the well-characterized AM251 anorectic effect (Méndez-Díaz et al., 2012; Tallett et al., 2009). This finding reveals that both antagonists have the same latency to affect food ingestion. Apparently, SR141716 is less potent than the other antagonists at this equimolar dose, because the latency to significantly decrease food ingestion is about 4 h. The higher ENP11 (3 mg/kg) dose used to decreased food ingestion induced this effect for the following 24 h after administration, as AM251 (1.2 mg/kg) did. In short, ENP11 exhibited, at equimolar dose, a shorter latency than SR141716A and shorter half-life than AM251 to decrease food ingestion. This ENP11 short half-life seems to be convenient due to the fact that in some therapeutic circumstances a limited effect is desirable.

In addition, since ENP11 (1 mg/kg) antagonized AEA's orexigenic effect just as SR141716A and AM251 did (Colombo et al., 1998; Martínez-González et al., 2004; Méndez-Díaz et al., 2012), we believe

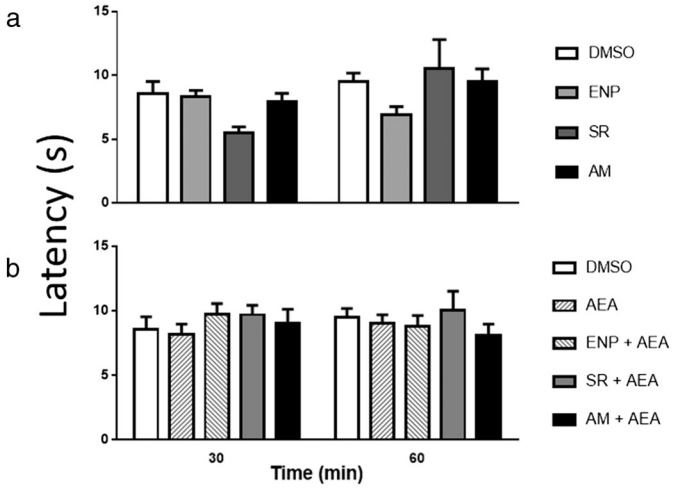

Fig. 3. Pain perception. The administration of antagonists (ENP 11, SR141716A and AM251), AEA or both, does not affect pain perception.

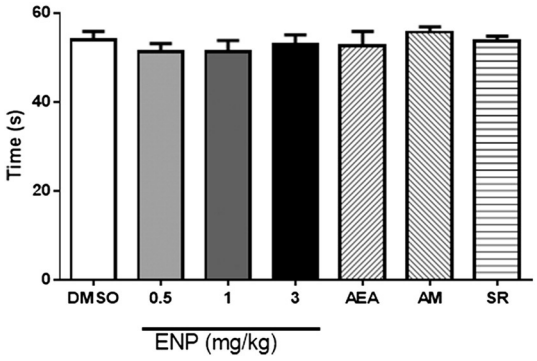

Fig. 4. Motor control. None of the treatments used affected motor control compared to DMSO.

that this drug also binds to CB1R. As we have previously demonstrated that ENP11 causes hypoglycemia in rats, it is very likely that the food ingestion reduction contributes to induce such hypoglycemia.

None of the antagonists used, at equimolar doses, affected the core temperature by themselves. But all of them, ENP11 included, prevented AEA's hypothermic effect (Fig. 2b). Moreover, none of these antagonists modified pain threshold (Fig. 3) or motor control (Fig. 4). Hence, we have gathered some evidence suggesting that ENP11 reduces food intake (even for 24 h at the higher dose used in this study), without inducing side effects on pain perception or motor control, suggesting it as a therapeutic option to decrease food ingestion. However, further experiments assessing its potential to induce depression-like symptoms will complement the present findings.

5. Conclusion

This study sheds some evidence supporting ENP11's potential anorexigenic properties. Moreover, ENP11's capacity to block some AEA effects supports its interaction with the CB1R.

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