Brief Communication

MOLECULAR METABOLISM

mTORC1 and CB1 receptor signaling regulate excitatory glutamatergic inputs onto the hypothalamic paraventricular nucleus in response to energy availability

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ABSTRACT

Objective: The hypothalamic paraventricular nucleus (PVN) is a key target of the melanocortin system, which orchestrates behavioral and metabolic responses depending on energy availability. The mechanistic target of rapamycin complex 1 (mTORC1) and the endocannabinoid type 1 receptor (CB1R) pathways are two key signaling systems involved in the regulation of energy balance whose activity closely depends upon energy availability. Here we tested the hypothesis that modulation of mTORC1 and CB1R signaling regulates excitatory glutamatergic inputs onto the PVN.
Methods: Patch-clamp recordings in C57BL/6J mice, in mice lacking the mTORC1 component *Rptor* or *CB1R* in pro-opio-melanocortin (POMC) neurons, combined with pharmacology targeting mTORC1, the melanocortin receptor type 4 (MC4R), or the endocannabinoid system under chow or a hypercaloric diet.
Results: Acute pharmacological inhibition of mTORC1 in C57BL/6J mice decreased glutamatergic inputs onto the PVN *via* a mechanism requiring modulation of MC4R, endocannabinoid 2-AG mobilization by PVN parvocellular neurons, and retrograde activation of presynaptic CB1R. Further electrophysiology studies using mice lacking mTORC1 activity or CB1R in POMC neurons indicated that the observed effects involved mTORC1 and CB1R-dependent regulation of glutamate release from POMC neurons. Finally, energy surfeit caused by hypercaloric high-fat diet feeding, rapidly and time-dependently altered the glutamatergic inputs onto parvocellular neurons and the ability of mTORC1 and CB1R signaling to modulate such excitatory activity.
Conclusions: These findings pinpoint the relationship between mTORC1 and endocannabinoid-CB1R signaling in the regulation of the POMC-mediated glutamatergic inputs onto PVN parvocellular neurons and its rapid alteration in conditions favoring the development of obesity.

Keywords mTOR; CB1 receptor; Glutamate; Hypothalamus; High-fat diet

1. INTRODUCTION

The paraventricular nucleus (PVN) of the hypothalamus plays key roles in the regulation of energy balance, the general response to stress, and the control of hypothalamic-peripheral hormonal axes [1–3]. PVN neurons expressing melanocortin receptors are the target of both Pro-opio-melanocortin (POMC)- and agouti-related protein (AgRP)-expressing neurons of the hypothalamic arcuate nucleus (ARC), forming the hypothalamic melanocortin system, a circuit critical for the regulation of energy balance [4,5]. In particular, the POMC-derived melanocortin peptide α-melanocyte stimulating hormone (α-MSH) decreases food intake and stimulates energy expenditure through its binding to melanocortin receptor type 4 (MC4R) [6]. Intra-PVN administration of α-MSH or its analogs reduces feeding, while MC4R antagonists, including AgRP, increase feeding [7–10]. Accordingly, re-expression of MC4R in the PVN of MC4R-null obese mice reduces excessive food intake [6]. Consumption of food also activates POMC neurons, which, in turn, activate parvocellular neurons of the PVN to modulate food intake [11–13]. Thus, PVN parvocellular neurons are recruited to link the detection of energy availability to consequent behaviors. MC4R-expressing neurons, including parvocellular neurons, are glutamatergic [12,14,15], and selective disruption of glutamate release from MC4R PVN neurons causes hyperphagia and obesity [14,15]. Consequently, better understanding of how the activity of PVN parvocellular neurons is regulated may help unveil mechanisms going awry in obesity. In addition to releasing neuropeptides [16], POMC neurons can also release neurotransmitters like glutamate and/or GABA [17–19]. However, whether excitatory glutamatergic transmission from POMC neurons affects the activity of PVN parvocellular neurons is not known.

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The mechanistic target of rapamycin (mTOR) kinase is an important intracellular energy sensor forming two distinct complexes (mTOR complex 1 or mTORC1, and mTOR complex 2 or mTORC2), controlling cellular metabolism in response to nutrients, growth factors, mitogens, hormones, and cellular stress [20,21]. Increased mTORC1 activity is associated with increased intracellular energy availability [20,21]. We and others have shown that mTORC1 signaling participates in the regulation of energy balance by modulating the function of both POMC and AgRP neurons [22–26]. mTORC1 activity also regulates oxidative metabolism in POMC neurons, which, in turn, is critical for the appetite suppressant action of the hormone leptin [27]. However, whether mTORC1 activity in POMC neurons affects neurotransmitter release, particularly onto PVN parvocellular neurons, is currently unknown.

Differently, the endocannabinoid system, including the cannabinoid receptor type 1 (CB1R) and endogenous lipid ligands called endocannabinoids, is known to modulate neurotransmission through the binding of endocannabinoids to presynaptic CB1R in different brain regions [28,29]. Opposite to what is observed for mTORC1 activity, endocannabinoids levels are physiologically increased in conditions of negative energy balance, such as fasting, and decreased after food consumption or in response to signals of positive energy balance, such as leptin [30,31]. Moreover, CB1R activity in hypothalamic neurons affects food intake and metabolic responses in a cell-type specific manner [32–36].

CB1R signaling can affect the mTOR pathway in different brain regions [37]. Here, we investigated the putative link between mTORC1 and CB1R signaling in the modulation of excitatory glutamatergic neurotransmission at the synapse between hypothalamic POMC and parvocellular neurons. We specifically studied changes in glutamatergic miniatures excitatory post-synaptic currents (mEPSC), which are an indicator of the glutamatergic inputs received by a cell, and thus cell excitability [38]. Consequently, in the context of our studies, the higher the mESPC frequency, the higher the probability that the parvocellular neuron-dependent anorexigenic-related signal will pass onto the next cell. Conversely, conditions reducing mEPSC frequency would limit the propagation of such signal.

The results show that mTORC1 activity in POMC neurons regulates glutamatergic inputs to PVN parvocellular neurons and that this function is exerted *via* control of pre-synaptic CB1R. Importantly, conditions of energy surfeit, as upon hypercaloric high-fat diet (HFD) feeding, inhibit excitatory glutamatergic input onto parvocellular neurons through a time-dependent modulation of mTORC1 and CB1R activity.

2. MATERIAL AND METHODS

2.1. Animals

The experiments were conducted in strict compliance with European Union Directives (2010/63/EU) and were approved by the ethical committee of the University of Bordeaux (DIR1354). All procedures followed the ARRIVE guidelines [39].

POMCCre$^{+/+}$::CB1R$^{flox/flox}$ mice (lacking the expression of CB1R in POMC cells and thereafter called POMC-*CB1R*-KO) and their POMCCre$^{-/-}$::CB1R$^{flox/flox}$ control littermates were generated by crossing POMC-Cre mice [40] (Tg(Pomc1-cre)16Lowl/J, JAX Stock #005965, The Jackson Laboratory, USA) with CB1R-flox mice [41]. This mouse line was generated following a 3-step backcrossing method [42]. POMC-*Rptor*-KO and their control littermates, which were previously characterized [27], C57BL/6J mice (Janvier, France) and whole body *CB1R*-KO [41] were also used (see Supplementary Methods). Conditional mouse lines were genotyped by PCR [42] using specific primers (Suppl. Table 1).

Two-to 3- month-old male mice were housed individually under a 12 h light/dark cycle (lights on at 01:00 am), at 22 ± 2 °C. They had *ad libitum* access to water and chow (Standard Rodent Diet A03, 3.2 kcal/g; SAFE, France) or HFD (60% of calories from fat; D12492 Research Diets, New Brunswick, NJ), which was given for a period of 1 or 4 weeks, to provide temporal information concerning the adaptive changes of the circuit over time. The number of animals used and related number of neurons studied are detailed in the figure legends.

2.2. Electrophysiology studies

The brain slices preparation is described in the Supplementary Methods.

Patch-clamp recordings. Patch electrodes were pulled (micropipette puller P-97, Sutter instrument, USA) from borosilicate glass (O.D. 1.5 mm, I.D. 0.86 mm, Sutter Instrument) to a resistance of 2–4 mΩ. Electrophysiological data were recorded using a Multiclamp 700B amplifier (Molecular devices, UK), low-pass filtered at 4 kHz and digitized at 10 Hz (current clamp) or 4 Hz (voltage clamp) (Digidata 1440A, Molecular devices, UK). Signals were analyzed offline (Clampfit software, pClamp 10, Molecular devices, UK).

The pipette internal solution used of mEPSCs acquisition contained [in mM: 125 potassium gluconate, 5 KCl, 10 Hepes, 0.6 EGTA, 0.3 $CaCl_2$, 2 $MgCl_2$, 7 Phosphocreatine, 3 adenosine-5′-triphosphate (magnesium salt), 0.3 guanosine-5′-triphosphate (sodium salt) (pH adjusted to 7.25 with KOH; osmolarity 300 mOsm/l adjusted with d-Mannitol; liquid junction potential −14.8 mV corrected on the data presented)]. In the PVN, parvocellular neurons were differentiated from magnocellular neurons immediately after patch rupture by preliminary electrophysiological analysis. Indeed, when submitted to a depolarizing current, magnocellular neurons exhibit a large transient outward rectification, not found in parvocellular neurons [43]. Then cells were switched in voltage clamp (V_{hold} = −70 mV).

Miniature glutamatergic transmission was pharmacologically isolated using a $GABA_A$ selective antagonist (picrotoxin, 100 μM, Sigma Aldrich, France) and voltage-gated sodium channel inhibitor tetrodotoxin (TTX, 1 μM, Tocris, France), which blocks the spontaneous activity of the network. As a control, perfusion of a mix of glutamatergic transmission antagonists [NBQX (10 μM), APV (50 μM), both from Abcam, France] at the end of the experiment fully blocked miniature transmission.

Implication of mTORC1 signaling was tested by adding rapamycin (200 nM, VWR International, France) in the perfusion bath for 5 min. To challenge CB1R signaling, a selective agonist (WIN55-212, 5 μM, from Tocris) or antagonist (AM251, 4 μM, from Tocris) was added for at least 12 min. MC4R were pharmacologically activated with the α-MSH analog melanotan II (MTII, 100 nM). Addition of the calcium chelator BAPTA (10 mM, Sigma–Aldrich) to the intracellular recording solution allowed inhibiting endocannabinoid synthesis [28,44] in the recorded parvocellular neuron. Frequency was evaluated after a stabilization period of at least 10 min after the addition of BAPTA. Then, pre-incubation of the slices with a Tetrahydrolipstatin (THL, 25 μM, Sigma Aldrich), an inhibitor of the endocannabinoid 2-arachidonoylglycerol (2-AG) synthesis enzyme diacylglycerol lipase [45,46], for at least 30 min before and throughout the assay, specifically blocked the synthesis of 2-AG [45,46].

2.3. *Double fluorescent* in situ *hybridization*

The details about the mice used and the tissue collection, the generation of the riboprobes, the signal amplification, and neuroanatomical analysis are provided in the Supplementary Methods.

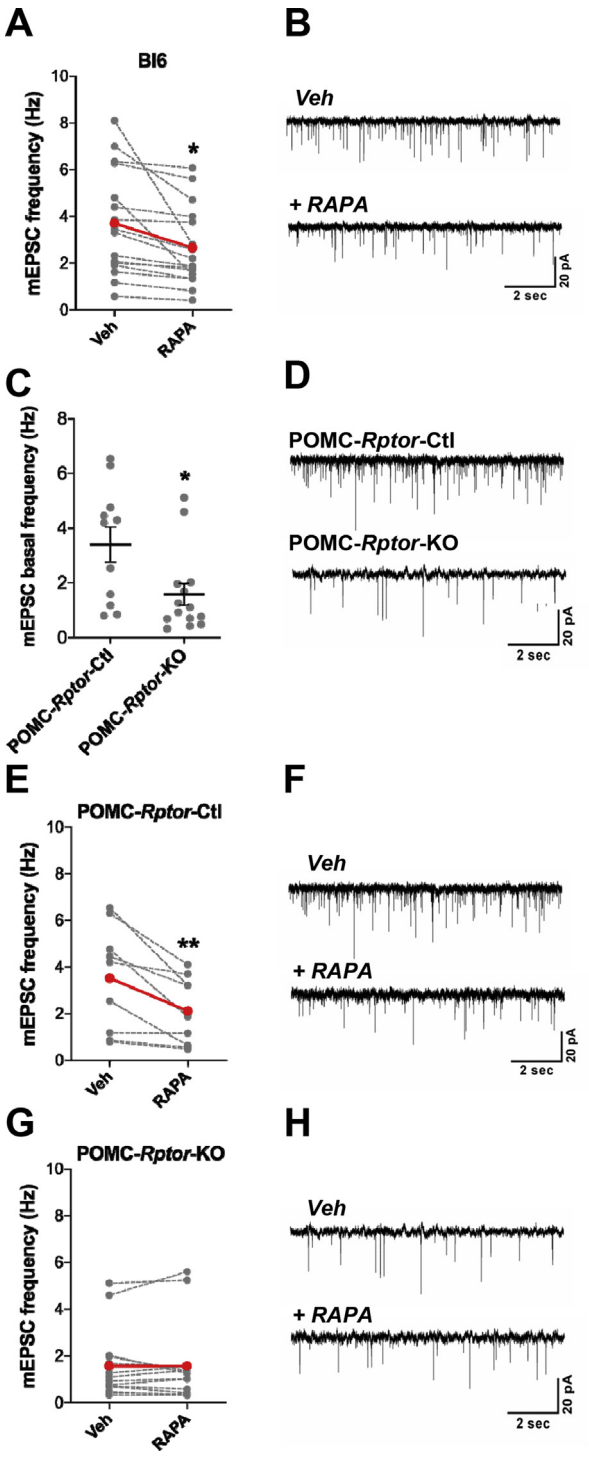

Figure 1: mTORC1 activity in POMC neurons regulates glutamate release onto PVN parvocellular neurons. mTORC1 blockade with rapamycin (RAPA) significantly reduces mEPSC frequency in BI6 (**A**, **B**; $t_{(15)} = 2.930$, $p = 0.0103$; 16 cells from 5 mice). POMC-*Rptor*-KO mice have reduced mEPSC frequency as compared to their control littermates (**C**, **D**; $t_{(23)} = 2,232$, $p = 0.018$; 11 cells from 3 control animals, 14 cells from 4 KOs). RAPA reduces mEPSC frequency in POMC-*Rptor*-control mice (**E**, **F**; $t_{(8)} = 3.464$ $p = 0.008$; 9 cells from 3 mice) but has no effect in POMC-*Rptor*-KO littermates (**G**, **H**; $t_{(13)} = 0.087$, $p = 0.93$; 14 cells from 4 mice). Frequency of individual cells tested before (vehicle, veh) and after rapamycin (RAPA) (A, C, E and G), with representative mEPSC traces (B, D, F and H). Grey lines are cells' individual behavior; red line is the mean for the tested condition. *$p < 0.05$, **$p < 0.01$.

2.4. Statistics

Values are mean ± SEM. Miniature events frequency and amplitude during the last 4 min of baseline were compared to the same parameters after 10 min of drug perfusion (4 min also) using a student t-test or paired t-test with GraphPad version 8 (La Jolla, USA). Diet effect was analyzed using one-way ANOVA followed by Tukey's post-hoc test. Treatment and diet effects and their interaction in matched samples were analyzed using two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. $P < 0.05$ denotes statistical significance.

3. RESULTS

3.1. mTORC1 activity controls excitatory glutamatergic POMC inputs onto PVN parvocellular neurons

To investigate the role of mTORC1 in the regulation of glutamatergic inputs onto PVN parvocellular neurons, we applied the mTORC1 inhibitor rapamycin during recording. This treatment rapidly reduced mEPSC frequency (−28.2%, Figure 1A,B), suggesting a change in presynaptic release mechanisms [38], without modifying events amplitude (Suppl. Figure 1A), which would instead indicate a change in postsynaptic functions [38]. As PVN neurons receive glutamatergic inputs from various brain regions [47,48] and mEPSC recording does not allow for identifying the exact source of the neurotransmitter, the effect of rapamycin could be due to mTORC1 inhibition in different PVN afferent cells. To test whether the observed changes depended upon mTORC1 activity specifically in POMC neurons, the same experiments were performed in POMC-*Rptor*-KO mice, which lack functional mTORC1 in POMC neurons [27] and their control littermates. POMC-*Rptor*-KO mice had reduced mEPSC frequency similar to what observed with rapamycin (Figure 1C,D), indicating that functional mTORC1 in pre-synaptic POMC neurons is necessary to assure normal excitatory drive of PVN parvocellular neurons. Other afferents onto PVN neurons might still be involved in the effect on mEPSC observed with rapamycin. However, while rapamycin significantly decreased mEPSC frequency in controls (−40.1%, Figure 1E,F), it had no effect in POMC-*Rptor*-KO (Figure 1G,H). Considering that none of these experiments induced changes in mEPSC amplitude (Suppl. Figure 1 B–D), these data indicate that presynaptic mTORC1 activity in POMC neurons is required to maintain basal levels of glutamate release onto PVN parvocellular neurons.

3.2. *Acute mTORC1 inhibition affects glutamate release* via *CB1R signaling*

Endocannabinoid-CB1R signaling is a typical pre-synaptic mechanism, the activation of which reduces neurotransmitter release [28,29]. To test whether CB1R might mediate the effect of rapamycin on mEPSC frequency, we blocked CB1R signaling. The application of the selective CB1R antagonist AM251 in the presence of rapamycin, re-established basal mEPSC frequency, without changes in amplitude (Figure 2A,B and Suppl. Figure 2A). As CB1R is expressed in POMC neurons [36], we generated mice lacking CB1R in POMC cells (thereafter called POMC-*CB1R*-KO). These animals had a specific deletion of *CB1R* in POMC neurons (Figure 2C), while expression of CB1R was fully preserved in AgRP cells (Figure 2D) and in other brain areas (data not shown).

Pharmacological blockade of mTORC1 decreased mEPSC frequency in controls (−30.9%, Figure 2E,F), but not in POMC-*CB1R*-KO mice (Figure 2G,H), indicating that activation of CB1R in POMC neurons is necessary to observe the effect of rapamycin on mEPSC frequency. None of these experiments revealed any alteration of mEPSC amplitude (Suppl. Figure 2 B–D). In agreement with a role of CB1R signaling in

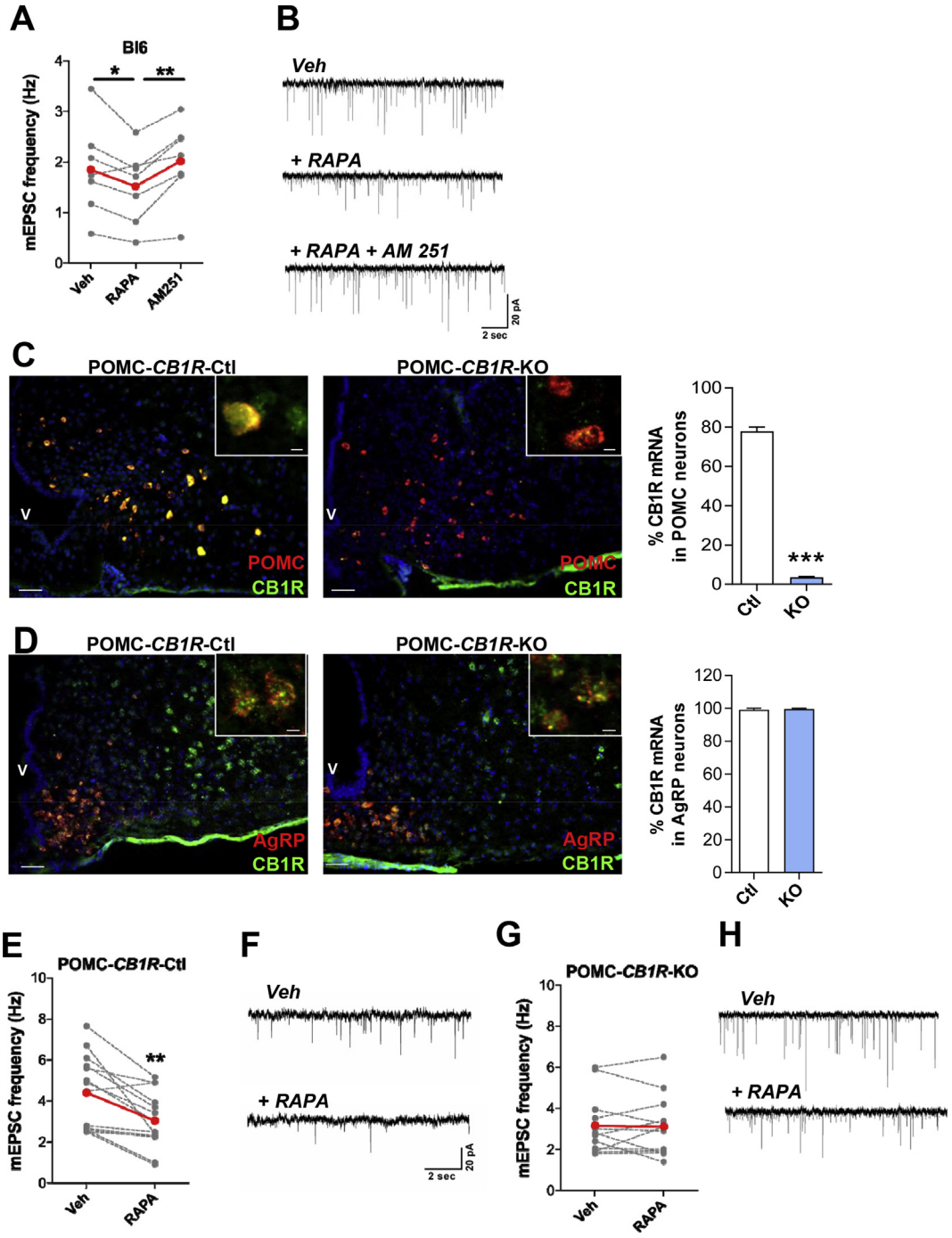

Figure 2: CB1 receptors in POMC neurons are necessary for mTORC1-dependent regulation of glutamate release onto PVN parvocellular neurons. In Bl6 mice, RAPA-induced reduction in mEPSC frequency is rescued by the CB1R antagonist AM251 (**A**, **B**; Veh *vs* RAPA: $t_{(6)} = 2.761$, $p = 0.03$; RAPA *vs* AM251: $t_{(6)} = 4.541$, $p = 0.004$; 7 cells from 2 mice). (**C**, **D**) Representative images taken at the level of the ARC and related quantification of fluorescent *in situ* hybridization studies showing colocalization of CB_1R mRNA expression (green color) in either POMC mRNA-positive neurons (red color, C) or AgRP mRNA-positive neurons (red color, D) of POMC-*CB_1*-control and their KO littermates (for POMC analysis in C: $t_{(4)} = 33.212$ $p < 0.0001$; for AgRP analysis in D: $t_{(4)} = 0.511$ $p = 0.636$; $n = 3$ mice per group). Nuclear counterstain carried out with DAPI. Scale bar in C and D: 50 μm, and 5 μm for smaller inset. V: 3rd ventricle. RAPA decreases mEPSC frequency in POMC-*CB1R*-Control mice (**E**, **F**; $t_{(13)} = 4.171$, $p = 0.001$; 14 cells from 7 mice), but it has no effect in POMC-*CB1R*-KO littermates (**G**, **H**; $t_{(11)} = 0.216$, $p = 0.83$; 12 cells from 5 mice). Representative mEPSC traces (B, F, H). Grey lines are cells' individual behavior; red line is the mean for the tested condition. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$.

the modulation of mEPSC frequency at PVN parvocellular neurons, we observed that pharmacological activation of CB1R with the CB1R agonist WIN55,212-2 decreased mEPSC frequency (Figure 3A,B). This effect was blunted in POMC-*CB1R*-KO mice (Figure 3C,D), suggesting that the majority of CB1R-dependent control of mEPSC onto parvocellular cells depends on the presence of the receptor in POMC neurons. In none of these experiments we observed significant changes in mEPSC amplitude (Suppl. Figure 3 A and B).

Activation of presynaptic CB1R generally occurs *via* retrograde calcium-dependent mobilization of lipid endocannabinoids from post-

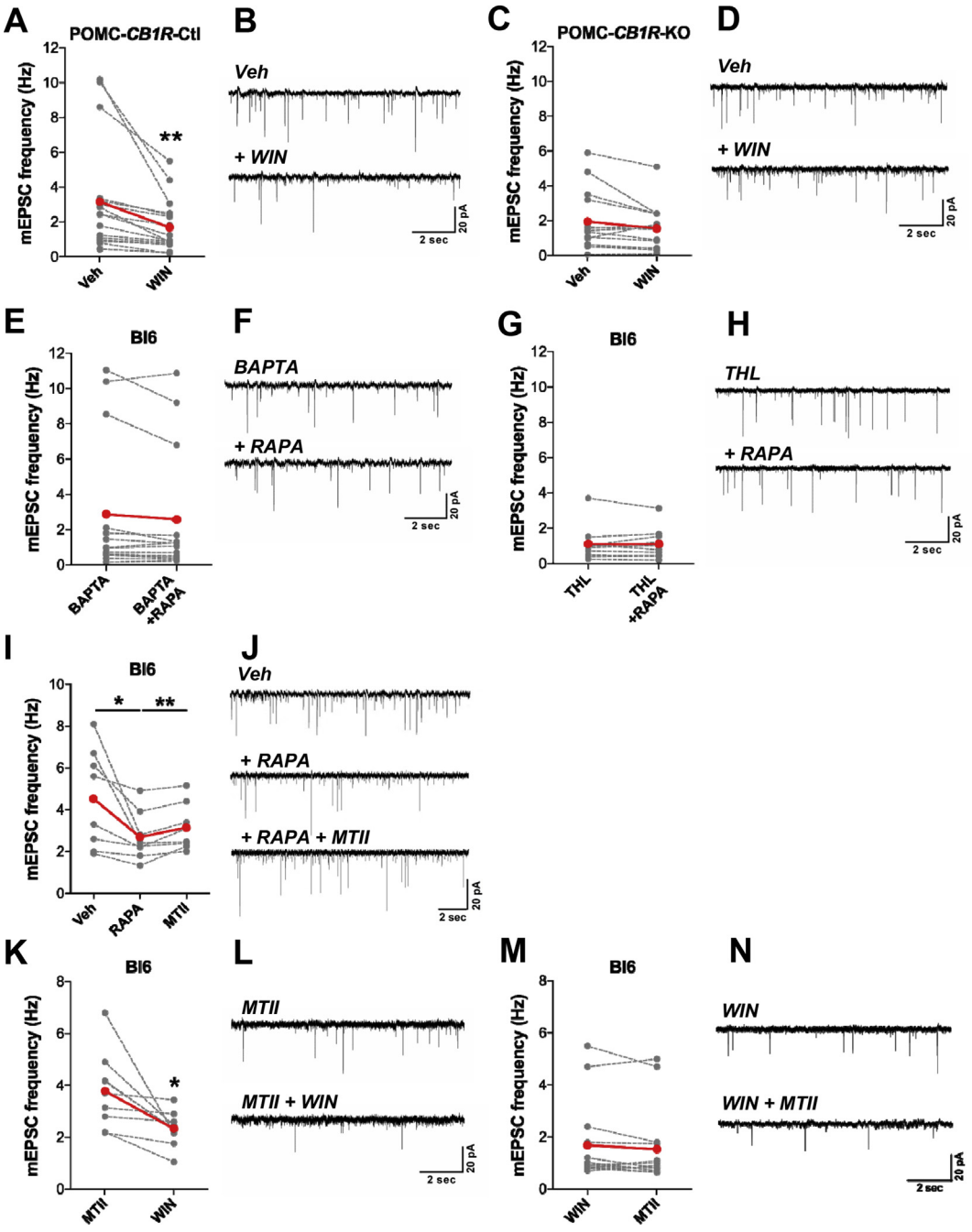

Figure 3: Inhibitory action of rapamycin on glutamate release onto PVN parvocellular neurons requires endocannabinoids synthesis in the postsynaptic parvocellular cell. In POMC-*CB1R*-Control mice, application of the CB1R agonist WIN55-212,2 reduces mEPSC frequency (**A**, **B**; $t_{(16)} = 3.014$, $p = 0.008$; 17 cells from 7 mice) while it has no effect in POMC-*CB1R*-KO littermates (**C**, **D**; $t_{(13)} = 1.927$, $p = 0.07$; 14 cells from 5 mice). RAPA has no effect when it is applied after BAPTA (**E**, **F**; $t_{(13)} = 1.638$, $p = 0.12$; 14 cells from 4 mice) or THL (**G**, **H**; $t_{(9)} = 0.073$, $p = 0.94$; 10 cells from 3 mice) pre-incubation. RAPA-induced decrease in mEPSC frequency is partially rescued by the MC4R agonist MTII (**I**, **J**; Veh vs RAPA: $t_{(7)} = 2.654$, $p = 0.03$; RAPA vs MTII: $t_{(7)} = 3.736$, $p = 0.007$; 8 cells from 4 mice). When MC4R are saturated with MTII, the CB1R agonist WIN still decreases mEPSCs frequency (**K**, **L**; $t_{(8)} = 2.983$, $p = 0.01$; 9 cells from 3 mice). When CB1R are saturated with WIN, MTII has no effect (**M**, **N**; $t_{(13)} = 1.698$, $p = 0.12$; 14 cells from 5 mice). Representative mEPSC traces (B, D, F, H, J, L, N). Grey lines are cells' individual behavior; red line is the mean for the tested condition. *$p < 0.05$, **$p < 0.01$.

synaptic cells [28,29]. Therefore, we assessed whether post-synaptic calcium levels played a role in the effect of rapamycin on mEPSC frequency. The infusion of the calcium chelator BAPTA into PVN parvocellular neurons *via* the patching pipette fully blocked the rapamycin-induced decrease of mEPSC frequency (Figure 3E,F), indicating the involvement of a calcium-dependent post-synaptic mechanism. Most endocannabinoids are lipid molecules, and the two derivatives of arachidonic acid anandamide and 2-AG are the best characterized [49,50]. To assess the nature of the endocannabinoid involved in the effect of rapamycin, we applied the 2-AG synthesis inhibitor THL [45], which prevented the rapamycin-induced reduction of mEPSC frequency (Figure 3G,H), without any change in amplitude (Suppl. Figure 3C, D). Thus, acute mTORC1 inhibition mobilizes post-synaptic endocannabinoids (likely 2-AG) to activate pre-synaptic CB1R

on POMC neurons to eventually reduce glutamate release onto PVN parvocellular cells. Finally, we evaluated the involvement of post-synaptic MC4R in this context, by using the α-MSH analog MTII. MC4R activation *per se* did not affect mEPSC frequency or amplitude (Suppl. Figure 3 E, F). However this manipulation partially restored rapamycin-induced decrease in mEPSC frequency (Figure 3I,J). To then assess the temporal relation between post-synaptic MC4R and pre-synaptic CB1R in the modulation of glutamate release, we combined MTII with the CB1R agonist WIN 55,212-2. After MTII pre-incubation, CB1R activation reduced mEPSC frequency (Figure 3K,L), while in the presence of WIN (saturated CB1R), MTII had no effect (Figure 3M,N). In all these studies there were no changes in mEPSC amplitude (Suppl. Figure 3G—I). These data therefore suggest that pre-synaptic CB1R activation is downstream the modulation of post-synaptic melanocortin signaling.

3.3. Effect of HFD exposure on mEPSC frequency of parvocellular neurons

Both hypothalamic mTORC1 and CB1R signaling are known to be altered by HFD feeding [50—53]. Hence, we wondered if HFD exposure could modify mEPSC frequency and how, in turn, modulation of mEPSC frequency by mTORC1 and CB1R signaling could be altered by obesogenic conditions.

One week of HFD dramatically decreased basal mEPSC frequency (−47.5%, Figure 4A), an effect that was maintained after 4 weeks of HFD (−48.7%, Figure 4A). Rapamycin decreased mEPSC frequency after 1 week, but not after 4 weeks of HFD (Figure 4B—F). No changes related to the type of diet or to the treatment were observed in mEPSC amplitude (Suppl. Figure 4A). We then tested the involvement of CB1R signaling in the rapamycin-induced changes in mEPSC frequency under HFD. After 1 week of HFD and similarly to what reported in chow (Figure 2A,B), incubation with the CB1R antagonist AM251 rescued rapamycin effect on mEPSC frequency, with no effect on mEPSC amplitude (Figure 4C,D and Suppl. Figure 4B). However, rapamycin and AM251 did not alter mEPSC frequency or amplitude after 4 weeks (Figure 4E,F and Suppl. Figure 4C). Finally, we evaluated the time course effect of the diet on the action of AM251-or WIN-induced modulation of mEPSC frequency. As expected, in chow, AM251 did not alter mEPSC frequency, but after 1 week of HFD, it rescued the low mEPSC frequency induced by HFD and had again no effect after 4 weeks of HFD (Figure 4G). Conversely, application of WIN overall decreased mEPSC frequency, with an action particularly evident under chow as compared to HFD (Figure 4H). However, under these conditions, a small mEPSC amplitude effect of AM251 (Suppl. Figure 4D) and the diet (Suppl. Figure 4E) were observed.

Overall, these data suggest the existence of an endocannabinoid tone modulating excitatory glutamatergic transmission onto PVN parvocellular neurons, whose action varies in relation to the type of diet and the length of exposure to the diet.

4. DISCUSSION

Here we have investigated how mTORC1 and endocannabinoid-CB1R signaling, molecular pathways known to work as readouts of cellular energy availability [21,50], impact excitatory glutamatergic neurotransmission onto PVN parvocellular neurons, which participate to the regulation of food intake and metabolic responses [6,12,14,15]. Our findings reveal that mTORC1 and CB1R signaling are critically involved in the modulation of glutamatergic inputs onto PVN parvocellular neurons, and that such action is exerted at the level of the POMC-parvocellular synapse.

Electrophysiological data obtained from C57BL/6J mice show that glutamatergic inputs onto parvocellular neurons are under the control of mTORC1 activity, since acute mTORC1 inhibition using rapamycin decreases mEPSC frequency. By blocking mTOR, rapamycin acutely increases food intake [22] and causes intracellular metabolic alterations mimicking a negative energy status [20]. Accordingly, this molecular change engages, through a still unknown mechanism, the mobilization from parvocellular neurons of endocannabinoids, which are signals of negative energy availability [50]. Our pharmacology studies using BAPTA and THL suggest that 2-AG is critically involved in determining the activation of presynaptic CB1R, hence inhibiting glutamatergic input onto parvocellular cells.

This response is logical in physiologic terms, as under conditions of negative energy availability, parvocellular cells activated by α-MSH are expected to be inhibited [14,15]. Accordingly, MC4R stimulation, which is associated with positive energy status [6], potentiates excitatory glutamatergic transmission onto PVN cells [54]. In line with this piece of evidence, MTII partially rescued rapamycin-induced decrease in mEPSC frequency and activation of pre-synaptic CB1R happened downstream of post-synaptic MC4R, overall implying that activation of MC4R at the parvocellular neuron may help inhibiting endocannabinoids mobilization. Furthermore, both POMC-*Rptor*-KO and POMC-*CB1R*-KO mice showed decreased mEPSC frequency at the parvocellular neurons, and use of these genetic models allowed demonstrating that the effect of rapamycin on mEPSC frequency depends on mTORC1 and CB1R in POMC neurons.

These findings have several implications. First, they suggest that there is a POMC-glutamatergic neurotransmission onto parvocellular neurons of the PVN. This evidence is in agreement with previous studies showing that POMC neurons can be functional glutamatergic neurons [17—19,55,56]. POMC glutamatergic activity seems relevant for the control of body weight, since animals lacking the glutamatergic transporter vGlut2 in POMC neurons are prone to develop diet-induced obesity [19]. However, other studies have not found any POMC-glutamatergic transmission onto the PVN when using optogenetic approaches, rather showing that such transmission is under the control of ARC vGlut2-expressing neurons, 44% of which are nevertheless POMC-positive cells [54]. These differences could be due to the genetic models used, interval of time between the intra-ARC administration of viral vectors carrying the expression of opsins and the actual optogenetic modulation of POMC projections in the PVN, and the total number and type of PVN target cells analyzed, as Fenselau et al. did not specifically evaluate glutamatergic transmission onto MC4R-expressing parvocellular neurons [54]. Moreover, optically evoked synaptic transmission might be different from ongoing tonic excitatory inputs as revealed by recording mEPSC.

Second, we observed an impact of *Rptor* and *CB1R* deletion from POMC neurons, respectively, on basal mEPSC frequency, and we cannot exclude that these changes in basal activity are due to compensatory mechanisms in these genetic models.

Third, when we exposed C57BL/6J mice to HFD, we described a drastic decrease in mEPSC frequency induced by the diet and a time-dependent loss in the ability of rapamycin to modulate excitatory input onto parvocellular neurons, which was, at least in part, dependent upon CB1R. Indeed, by investigating mEPSC changes in response to CB1R pharmacological modulation, we could conclude that there is an endocannabinoid tone regulating glutamatergic transmission onto parvocellular neurons, which varies in relation to the length of exposure to the HFD. In particular, while changes in mEPSC frequency could still be observed after 1 week of HFD in response to mTOR and CB1R blockade (Figure 4C), this was not anymore the case after 4 weeks of

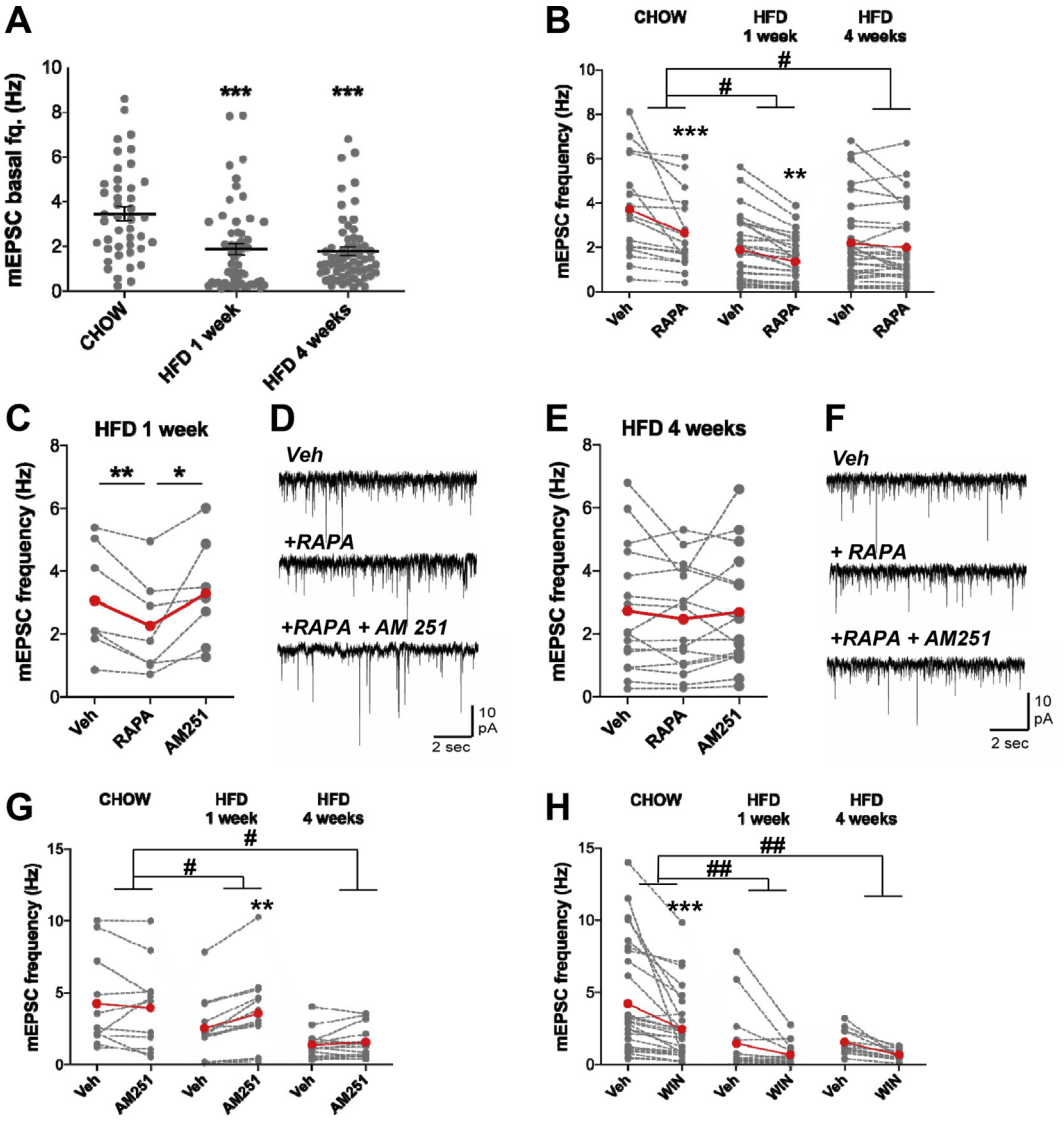

Figure 4: HFD exposure impacts glutamatergic inputs onto PVN parvocellular neurons and affects mTORC1 and endocannabinoid-CB1R dependent glutamate release. Basal mEPSC frequency is significantly reduced after 1 week and 4 weeks of HFD (**A**; diet effect $F_{(2,159)} = 13.15$, $p < 0.0001$; 43 cells from 14 mice in chow, 57 cells from 17 mice 1 week HFD, 62 cells from 15 mice 4 weeks HFD). RAPA perfusion significantly reduces mEPSC frequency under chow and after 1 week of HFD, but it has no effect after 4 weeks of HFD (**B**; diet effect $F_{(2,\ 71)} = 4.784$, $p = 0.011$, treatment effect $F_{(1,71)} = 33.52$, $p < 0.0001$, interaction $F_{(2,71)} = 5.038$, $p = 0.009$; 16 cells from 5 mice in chow, 28 cells from 11 mice 1 week HFD, 30 cells from 10 mice 4 weeks HFD). After 1 week of HFD, RAPA-induced mEPSC frequency reduction can be restored by application of the CB1R antagonist AM251 (**C**; Veh *vs* RAPA: $t_{(6)} = 3.873$, $p = 0.008$; RAPA *vs* AM251: $t_{(6)} = 2.564$, $p = 0.043$; 7 cells from 4 mice), whereas none of the compounds have an effect in animals fed with HFD for 4 weeks (**E**; Veh *vs* RAPA: $t_{(15)} = 1.309$, $p = 0.21$; RAPA *vs* AM251: $t_{(15)} = 0.959$, $p = 0.36$; 16 cells from 9 mice). Representatives mEPSC traces (D, F). AM251 has no effect on mEPSC frequency under chow, while it induces a significant increase of mEPSC frequency after 1 week of HFD and has again no effect after 4 weeks of HFD (**G**; diet effect $F_{(2,\ 35)} = 4.39$, $p = 0.019$, treatment effect $F_{(1,35)} = 4.2$, $p = 0.048$, interaction $F_{(2,35)} = 6.586$, $p = 0.004$; 11 cells from 2 mice in chow, 13 cells from 6 mice 1 week HFD, 14 cells from 6 mice 4 weeks HFD). Perfusion with the CB1R agonist WIN55-212,2 significantly limits events frequency in animals fed chow but not HFD (**H**; diet effect $F_{(2,\ 54)} = 7.011$, $p = 0.002$, treatment effect $F_{(1,54)} = 16.82$, $p = 0.0001$, interaction $F_{(2,54)} = 1.481$, $p = 0.23$; 29 cells from 11mice in chow, 15 cells from 5 mice 1 week HFD, 13 cells from 5 mice 4 weeks HFD). Grey lines are cells' individual behavior; red line is the mean for the tested condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; #$p < 0.05$, ##$p < 0.01$ for diet effect.

HFD (Figure 4E), implying that effects might have been occluded by changes in mTOR and CB1R activity induced by the HFD. Supporting this interpretation, hypothalamic endocannabinoid levels are very high in mice after 1 week of HFD, returning slowly to basal, chow-like levels, after 8 weeks on the diet [53]. Remarkably, we observed that ability of AM251 to increase mEPSC at parvocellular neurons was particularly evident at 1 week of HFD (Figure 4G) and that, for the same time point, inhibitory action of WIN on mEPSC was blunted (Figure 4H). Besides, hypothalamic mTORC1 signaling is reduced of around 25% after 1 day of HFD and of almost 60% after 4 weeks of HFD [51], which could explain the inability of rapamycin to further affect mEPSC frequency at this time point (Figure 4B,E).

Altogether, these data allow drawing a parallel between what we described in chow and in HFD. Decreased glutamatergic input is observed both during acute inhibition of mTOR in chow, where rapamycin mimics an energy negative-like state, as well as, paradoxically, during HFD, under energy surfeit. Our findings and above reviewed studies suggest that 1 week HFD exposure increases endocannabinoid tone, while likely simultaneously decreasing mTORC1 signaling. These molecular changes induced in the hypothalamus within few days by

HFD consumption, resemble molecular changes induced by physiological fasting, a situation characterized by low hypothalamic mTORC1 activity and high hypothalamic endocannabinoid levels [22,31]. Thus, initial HFD intake may cause a molecular "fasting-like" signature, which probably helps explaining the hyperphagia accompanying the first few days of HFD feeding [57,58], observed by using the same type of HFD used here [57]. Further studies are needed to determine the role played by excitatory glutamatergic input onto parvocellular neurons of the PVN in the rapid behavioral changes associated with the consumption of HFD and consequent development of obesity.

CONTRIBUTION STATEMENT

WM, NS, VS, and AC performed the experiments and collected the data; WM, GM, FM, and DC analyzed and critically discussed the data; WM and DC conceptualized the studies and DC together with FM supervised the work; WM and DC wrote the manuscript. All authors have read, edited and approved the final version of the manuscript.

DUALITY OF INTEREST

The authors declare no competing financial interest in relation to the work described.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

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