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Original article

MOLECULAR METABOLISM

Adiponectin potentiates the acute effects of leptin in arcuate *Pomc* neurons

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ABSTRACT

Objective: Adiponectin receptors (*AdipoRs*) are located on neurons of the hypothalamus involved in metabolic regulation — including arcuate proopiomelanocortin (*Pomc*) and Neuropeptide Y/Agouti-related peptide *(NPY/AgRP*) neurons. AdipoRs play a critical role in regulating glucose and fatty acid metabolism by initiating several signaling cascades overlapping with Leptin receptors (*LepR*s). However, the mechanism by which adiponectin regulates cellular activity in the brain remains undefined.

Methods: In order to resolve this issue, we utilized neuron-specific transgenic mouse models to identify *Pomc* and *NPY/AgRP* neurons which express *LepRs* for patch-clamp electrophysiology experiments.

Results: We found that leptin and adiponectin synergistically activated melanocortin neurons in the arcuate nucleus. Conversely, *NPY/AgRP* neurons were inhibited in response to adiponectin. The adiponectin-induced depolarization of arcuate *Pomc* neurons occurred via activation of Phosphoinositide-3-kinase (PI3K) signaling, independent of 5′ AMP-activated protein kinase (AMPK) activity. Adiponectin also activated melanocortin neurons at various physiological glucose levels.

Conclusions: Our results demonstrate a requirement for PI3K signaling in the acute adiponectin-induced effects on the cellular activity of arcuate melanocortin neurons. Moreover, these data provide evidence for PI3K as a substrate for both leptin and adiponectin to regulate energy balance and glucose metabolism via melanocortin activity.

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Keywords Melanocortin; Obesity; Diabetes; Energy balance; Patch-clamp; Electrophysiology

1. INTRODUCTION

Obesity is characterized by a chronic imbalance between energy intake and expenditure [1]. Multiple factors including hormonal regulation, fuel availability, and behavior contribute to energy utilization [2]. In the central nervous system, neuropeptide Y/agouti-related peptide (NPY/AgRP) and proopiomelanocortin (*Pomc*) neurons receive and integrate information about energy availability via circulating adipokines/hormones [e.g. leptin and adiponectin] [3—5].

Leptin receptors (*LepR*s) in arcuate *Pomc* neurons are required and sufficient for the proper regulation of energy balance and glucose homeostasis (including systemic insulin sensitivity and hepatic glucose production) [6]. Similarly, adiponectin receptors (*AdipoRs*) play a critical role in regulating glucose and fatty acid metabolism in peripheral tissues [7—9]. *AdipoRs* are also located in the central nervous system including neurons of the hypothalamus involved in metabolic regulation such as arcuate *Pomc* and *NPY/AgRP* neurons [10,11]. However, the effect of adiponectin in the brain to regulate energy balance has been contentious, with studies showing differential effects on food intake [10,12,13] while increasing energy expenditure [14]. Importantly, adiponectin may be beneficial in the absence of leptin [8]. Moreover, adiponectin potentiated the effects of leptin on thermogenesis, body weight, fat, and V_{02} utilization, effects dependent upon melanocortin activity [13]. These data support an important regulation of metabolism

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by adiponectin which requires activity within the central nervous system including melanocortin neurons. Together, these observations highlight a potential melanocortin-dependent compensatory/additive role for *AdipoRs* in the absence/presence of leptin.

LepRs and AdipoRs initiate several overlapping signaling cascades including Janus kinase 2/Signal transducer and activator of transcription 3 (JAK2/STAT3), Insulin receptor substrate 1/2 (IRS1/2), Forkhead box protein O1 (FOXO1), AMP-activated protein kinase (AMPK), and phosphoinositol-3-kinase (PI3K) [15–18]. Of note, *PI3K* is required for the acute cellular effects of leptin in arcuate *Pomc* and *NPY/AgRP* neurons [5,19–22]. Additionally, *PI3K* in arcuate *Pomc* neurons is required for the acute effect of leptin to reduce food intake [20]. In contrast, much of the activity of adiponectin has focused on its cognate effects through AMPK resulting in little understanding of the signaling mechanisms including PI3K which may be required for the acute effects of adiponectin in the central nervous system.

In the current study, we tested the hypothesis that adiponectin and leptin may synergistically activate melanocortin neurons. To test this hypothesis we utilized transgenic and Cre-Lox technology to identify *NPY/AgRP* and *Pomc* neurons which express *LepRs*. We found that leptin and adiponectin modulate neuronal excitability of melanocortin neurons in an additive manner, an activity that requires *PI3K* signaling. Together, these data provide evidence for *PI3K* as a substrate for both leptin and adiponectin to regulate metabolism via melanocortin activity.

2. METHODS

2.1. Animals

All mice were housed under standard laboratory conditions (12 h on/off; lights on at 7:00 a.m.) and temperature-controlled environment with food and water available ad libitum. All experiments were performed in accordance with the guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Texas Institutional Animal Care and Use Committee. To identify POMC neurons with or without leptin receptors, we generated Pomc-hrGFP::LepR-cre::tdtomato (PLT) mice as previously described [23] anatomically restricted to the arcuate nucleus of the hypothalamus. NPY/AgRP neurons anatomically restricted to the arcuate nucleus of the hypothalamus were targeted from NPY-hrGFP mice [24].

2.2. Electrophysiology studies

2.2.1. Slice preparation and whole-cell recordings

Brain slices were prepared from young adult male mice (5–7 weeks old) as previously described [20,23,25]. Briefly, male mice were deeply anesthetized with i.p. injection of 7% chloral hydrate and transcardially perfused with a modified ice-cold artificial CSF (ACSF) (described below). The mice were then decapitated, and the entire brain was removed and immediately submerged in ice-cold, carbogen-saturated (95% O2 and 5% CO2) ACSF (126 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 1.25 mM NaH2PO4, 26 mM NaHCO3, and 5 mM glucose). For some experiments, slices were perfused with ACSF containing 0.5 mM or 3 mM glucose by replacing glucose with equiosmolar amounts of sucrose [26]. Coronal sections (250 μm) were cut with a Leica VT1000S Vibratome and then incubated in oxygenated ACSF at room temperature for at least 1 h before recording. The slices were bathed in oxygenated ACSF (32 °C–34 °C) at a flow rate of ~2 ml/min. All electrophysiology recordings were performed at room temperature.

The pipette solution for whole-cell recording was modified to include an intracellular dye (Alexa Fluor350 hydrazide dye) for whole-cell recording: 120 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM CaCl2, 1 mM MgCl2, and 2 mM MgATP, 0.03 mM Alexa Fluor 350 hydrazide dye (pH 7.3). Epifluorescence was briefly used to target fluorescent cells, at which time the light source was switched to infrared differential interference contrast imaging to obtain the whole-cell recording (Zeiss Axioskop FS2 Plus equipped with a fixed stage and a QuantEM:512SC electron-multiplying charge-coupled device camera). Electrophysiological signals were recorded using an Axopatch 700B amplifier (Molecular Devices), low-pass filtered at 2–5 kHz, and analyzed offline on a PC with pCLAMP programs (Molecular Devices). Membrane potential and firing rate were measured by whole-cell current clamp recordings from *Pomc* neurons in brain slices. Recording electrodes had resistances of 2.5–5 MΩ when filled with the K-gluconate internal solution. Input resistance was assessed by measuring voltage deflection at the end of the response to a hyperpolarizing rectangular current pulse steps (500 ms of −10 to −50 pA).

Leptin (100 nM; provided by A.F. Parlow, through the National Hormone and Peptide Program), adiponectin (10 nM; Phoenix pharmaceuticals), AdipoRon (0.1 μM–10 μM; Tocris) were added to the ACSF for specific experiments. Solutions containing drug were typically perfused for 5 min. A drug effect was required to be associated temporally with peptide application, and the response had to be stable within a few minutes. A neuron was considered depolarized or hyperpolarized if a change in membrane potential was at least 2 mV in amplitude. Neurons were voltage-clamped at −75 mV (for excitatory postsynaptic currents) and −15 mV (for inhibitory postsynaptic currents). Frequency and peak amplitude were measured by using the Mini Analysis program (Synaptosoft, Inc.)

2.3. Drugs

TTX, SKF96365, and 2-APB were obtained from Tocris; LY294002 (10 μM) was obtained from Calbiochem. All solutions were made according to manufacturer's specifications. Stock solutions of SKF96365, 2-APB, were made by dissolution in DMSO (Sigma). The concentration of DMSO in the external solution was <0.1%. Stock solutions of leptin were made by dissolution in D-PBS (Gibco). Stock solutions of TTX were made by dissolution in de-ionized water.

2.4. Analysis and statistics

A value of twice the mean peak-to-peak noise level for a given recording in control solutions was used as the detection limit for minimal PSC amplitude (*i.e.* typically 5–10 pA). For spontaneous EPSCs and IPSCs (sEPSCs and sIPSCs), at least 2 min of activity was examined to identify effects on amplitude and frequency distributions. Effects on spontaneous PSC frequency before and during drug application were analyzed within a recording using the Kolmogorov–Smirnov (K–S) test (a nonparametric, distribution-free goodness-of-fit test for probability distributions). Typically, 100–300 events were compared for each condition. Pooled results from responding cells were analyzed using a paired *t* test; multiple groups of cells were compared using ANOVA. Proportions of responding cells from different groups were analyzed using a χ^2 test of independence. Effects on spontaneous PSC amplitude were analyzed using a paired two-tailed *t* test. Results are reported as the mean ± sem unless indicated otherwise; where *n* represents the number of cells studied. Significance was set at $p < 0.05$ for all statistical measures.

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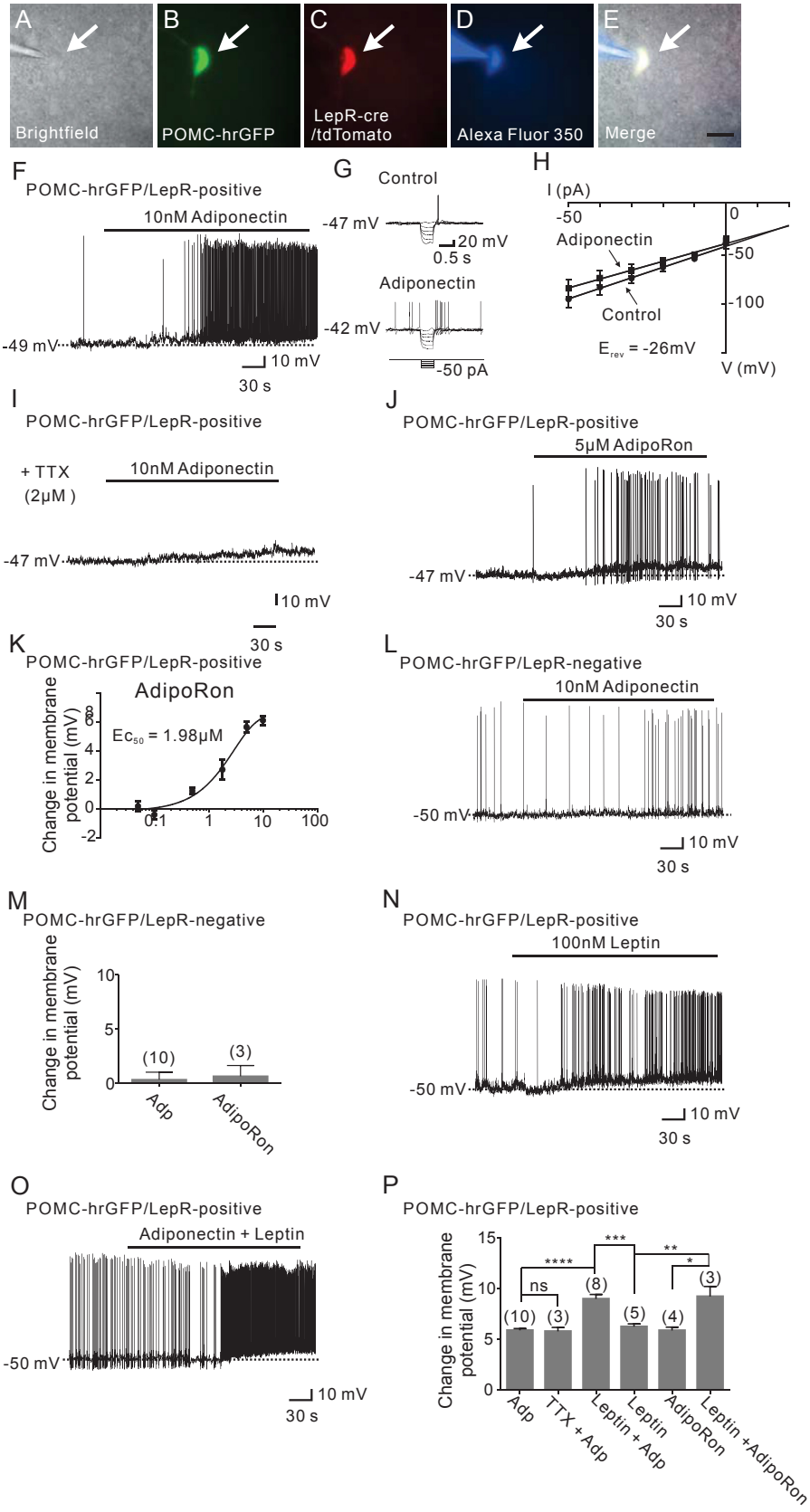

Figure 1: Adiponectin depolarizes *LepR*-positive *Pomc* neurons via activation of a mixed cation conductance. (A–E) Brightfield illumination (A) of *Pomc*-hrGFP::*LepR*-cre::tdtomato neuron from PLT mice. (B) and (C) show the same neuron under FITC (hrGFP) and Alexa Fluor 594 (tdtomato) illumination. Complete dialysis of Alexa Fluor 350 from the intracellular pipette is shown in (D) and merged image of targeted *Pomc* neuron (E). Arrow indicates the targeted cell. Scale bar = 50 μm. (F) Current-clamp record demonstrates a *Pomc*-

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3. RESULTS

3.1. Adiponectin depolarizes *LepR*-positive *Pomc* neurons via activation of a mixed cation conductance

In order to better identify adiponectin-induced effects on the membrane potential of *Pomc* neurons, whole-cell patch-clamp recordings were performed on *Pomc* neurons, which either express or do not express leptin receptors (*LepRs*) from *Pomc*-hrGFP::*LepR*-cre::tdtomato (PLT) mice [23,25]. *Pomc* neurons targeted for recording in the current study were anatomically restricted to the arcuate nucleus of the hypothalamus. Alexa Fluor350 hydrazide dye was added to the intracellular pipette solution for real-time confirmation that hrGFP-positive neurons were targeted for recording (Figure 1A–E). A majority of *LepR*-positive *Pomc* neurons were depolarized by local perfusion of adiponectin in a dose dependent manner (10 nM, 67%, $+5.9 \pm 0.2$ mV; resting membrane potential: -48.5 ± 2.4 mV; $n = 12$; Figure 1F,P and Supplemental Figure 1A,B). Application of adiponectin on *LepR*-positive *Pomc* neurons increased the action potential firing frequency (1.8 ± 0.5 Hz in normal ACSF; 5.1 ± 0.7 Hz in adiponectin; $n = 7$ Supplemental Figure 1B).

Adiponectin dose-dependently excites or inhibits neurons of the subfornical organ and the paraventricular hypothalamus [27–29]. However, the conductance involved has largely remained undefined. In order to better define the conductance, which contributes to the adiponectin-induced depolarization of *LepR*-expressing *Pomc* neurons, rectangular current steps (400 ms; ±50 pA) were applied to the membrane in order to obtain a current–voltage (*I–V*) plot. The depolarization was concomitant with a decrease in input resistance (27%, 1073 ± 54 MΩ for control, $n = 6$; 785 ± 95 MΩ for adiponectin, $n = 6$, Figure 1G). Subsequent linear regression analysis revealed the reversal potential of the adiponectin-induced depolarization to be -26.0 mV $\pm$ 5.1 mV ($n = 6$; Figure 1H), suggesting an activation of a mixed-cation conductance.

Importantly, pretreatment with TTX (2 uM) failed to abrogate adiponectin-induced depolarization of *Pomc* neurons (60%, $+5.8 \pm 0.4$ mV; resting membrane potential: -46.3 ± 1.2 mV; $n = 5$; Figure 1I,P), which supports a direct membrane depolarization independent of action-potential-mediated synaptic transmission. Analogous results were obtained using the synthetic small molecule agonist of the adiponectin receptors (AdipoRon). In particular, AdipoRon depolarized *LepR*-expressing *Pomc* neurons in a dose dependent manner (0.1–10 uM 5.6 $\pm$ 0.5 mV; resting membrane potential: -46.1 ± 2.2 mV; $n = 4$; Figure 1J,K,P, and Supplemental Figure 1B). Similar to the effects of adiponectin, the AdipoRon-induced depolarization was concomitant with an increase in the frequency of action potentials (5 μM; 2.0 ± 0.4 Hz in normal ACSF to 5.4 ± 0.9 Hz in AdipoRon, $n = 4$, Supplemental Figure 1B). Conversely, the majority (10 of 11) of *LepR*-negative *Pomc* neurons were unaffected by adiponectin (-0.4 ± 0.9 mV, 11 of 12 neurons, Figure 1L,M). The remaining 1 neuron was hyperpolarized (-6 mV). Together, these data suggest that the excitatory effects of adiponectin on *Pomc* neurons is largely selective to those which express leptin receptors (Figure 1M,P).

3.2. Adiponectin potentiates the leptin-induced excitation of *Pomc* neurons

Adiponectin rescued many of the metabolic deficits observed in ob/ob mice; an effect that required melanocortin signaling [8,13,30]. Moreover, adiponectin potentiates leptin's effect to stimulate thermogenesis, fatty acid oxidation, and insulin sensitivity [13]. Thus, we hypothesized that leptin and adiponectin may have additive effects on the excitability of arcuate *Pomc* neurons. Consistent with previous reports, nearly 83% of LepR-positive POMC neurons were depolarized and increased action potential firing in response to leptin superfusion (100 nM, $+6.2 \pm 0.3$ mV; resting membrane potential: -50.0 ± 2.9 mV; $n = 6$; Figure 1N,P and Supplemental Figure 1B). Notably, combined administration with adiponectin (10 nM) and leptin (100 nM) resulted in a larger depolarization of *LepR*-positive *Pomc* neurons (55%; $+8.9 \pm 0.4$ mV; resting membrane potential: -48.0 ± 2.3 mV; $n = 12$; $p < 0.05$; Figure 1O,P, and Supplemental Figure 1B). Similarly, application of AdipRon combined with leptin also resulted in a robust depolarization of *LepR*-positive *Pomc* neurons (75%; $+9.2 \pm 1.0$ mV; resting membrane potential: -47.4 ± 3.7 mV; $n = 4$; $p < 0.05$; Figure 1P and Supplemental Figure 1B). Combined administration of adiponectin or AdipoRon with leptin also resulted in the potentiation of the action potential frequency (for adiponectin + leptin: 2.0 ± 0.6 Hz in normal ACSF to 6.7 ± 1.0 Hz in adiponectin + leptin, $n = 8$; for AdipoRon + leptin: 1.7 ± 0.2 Hz in normal ACSF to 6.2 ± 1.2 Hz in AdipoRon + leptin, $n = 3$; Supplemental Figure 1B). These data suggest that adiponectin can enhance the leptin-induced excitatory actions on *LepR*-positive *Pomc* neurons (Figure 1P).

Glucose concentrations within the brain have reported between 0.1 and 5 mM [31–33]. Our data suggest that adiponectin excites *Pomc* neurons in 5 mM glucose. In order to examine the effect of adiponectin throughout this physiological range, extracellular glucose concentrations were lowered to 3 mM or 0.5 mM. Interestingly, adiponectin and leptin-induced depolarizations were preserved in both 3 mM and 0.5 mM extracellular glucose concentrations. For 3 mM glucose, adiponectin depolarized 57% of neurons recorded ($+5.8 \pm 0.4$ mV; from resting membrane potential of -52.4 ± 1.7 mV, $n = 7$; Supplemental Figure 2). Similarly leptin depolarized 57% of neurons recorded ($+5.5 \pm 0.5$ mV; from resting membrane potential of -51.4 ± 2.5 mV, $n = 7$; $p < 0.05$; Supplemental Figure 2). Moreover, combined administration with adiponectin (10 nM) and leptin (100 nM) led to an enhanced depolarization of *LepR*-positive *Pomc* neurons (67% of neurons targeted; $+8.8 \pm 0.3$ mV; from resting membrane potential of -53.6 ± 4.8 mV; $n = 6$; $p < 0.05$; Supplemental Figure 2). Similar results were obtained at 0.5 mM extracellular glucose with adiponectin depolarizing 83% of *LepR*-positive *Pomc* neurons ($+6.7 \pm 1.0$ mV; from a resting membrane

hrGFP::*Lepr*-cre::tdtomato (green/red) neuron that is depolarized in response to adiponectin. (G) Current-clamp recording from a *LepR*-expressing *Pomc* neuron showing a decreased voltage deflection in response to current injection after adiponectin application (10 nM). (H) Current versus voltage (I–V) relationship of the group of *LepR*-expressing *Pomc* neurons examined in response to adiponectin. (I) Representative trace showing that pretreatment with TTX (2 μM) does not abrogate adiponectin-induced depolarization of *LepR*-expressing *Pomc* neurons. (J) Application of AdipoRon induced a robust depolarization of *LepR*-expressing *Pomc* neurons. (K) Plot showing the concentration dependence of the response to adiponectin in *LepR*-expressing *Pomc* neurons. (L) Current-clamp recording demonstrates a *Pomc*-hrGFP::*LepR*-negative (green) neuron that does not respond to adiponectin. (M) Histogram illustrates the adiponectin or AdipoRon-induced change of membrane potential of *Pomc*-hrGFP::*LepR*-negative neurons from PLT mice. Error bars indicate SEM. (N) Current-clamp recording at resting membrane potential showing a leptin-induced (100 nM) depolarization of *LepR*-expressing *Pomc* neurons. (O) A representative trace illustrating that administration of adiponectin enhanced the leptin-induced depolarization of *LepR*-expressing *Pomc* neurons. (P) Histogram illustrates the adiponectin-induced change of membrane potential of *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neurons from PLT mice with or without Leptin and TTX. **$p < 0.001$. Error bars indicate SEM.

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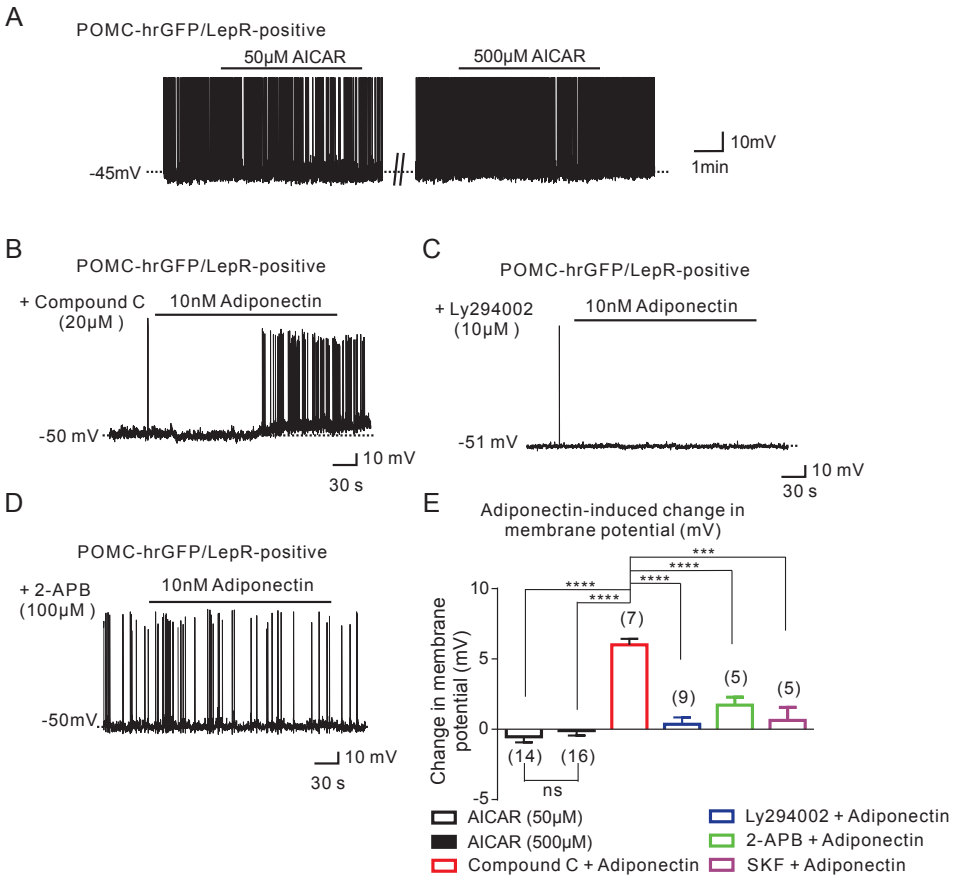

Figure 2: Adiponectin depolarizes *LepR*-positive *Pomc* neurons via *PI3K* mediated activation of *TrpC* channels. (A) Current-clamp recording at resting membrane potential demonstrating a *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron fails to respond to perfusion of the AMPK activator, AICAR (50 uM and 500 uM). (B) A representative trace shows that pretreatment with the AMPK inhibitor, compound C, does not abrogate the adiponectin-induced depolarization of *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron. (C) Current clamp recording demonstrates that pretreatment with the *PI3K* selective inhibitor LY294002 prevented the adiponectin-induced depolarization of *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron. (D) Pretreatment with the non-selective *TrpC* channel antagonist 2-APB blocks the adiponectin-induced depolarization of *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron. (E) Histogram illustrates the adiponectin induced change of membrane potential of *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron from PLT mice. **p $<$ 0.001. Error bars indicate SEM.

potential of -52.4 ± 2.3 mV; n $=$ 6; p $<$ 0.05; Supplemental Figure 2). Thus adiponectin stimulates arcuate *LepR*-expressing *Pomc* neurons independent of the proposed physiological glucose burden [31–33].

3.3. Adiponectin depolarized *LepR*-positive *Pomc* neurons via *PI3K* mediated *TRPC* channels

Several reports have highlighted the contribution of AMPK and PI3K to mediate adiponectin involved regulation of glucose metabolism and energy expenditure. However, the importance of AMPK or PI3K in the adiponectin-induced activation of *Pomc* neurons remains undefined. In order to better assess the requirement of AMPK in the adiponectin induced effects on membrane potential we used the AMPK activator AICAR. Several studies report an effective concentration of AICAR for various physiological processes including modification of electrophysiological cellular properties in the mid-micro molar range [34–36]. Perfusion of AICAR (50–500 μM) failed to alter the resting membrane potential of most *Pomc* neurons, which express leptin receptors (for 50 μM AICAR: 88%; -0.8 ± 0.3 mV; resting membrane potential: -46.4 ± 2.1 mV; n $=$ 14; p $>$ 0.05; for 500 μM AICAR: 100%; -0.1 ± 0.3 mV; resting membrane potential: -47.9 ± 1.9 mV; n $=$ 16; p $>$ 0.05; Figure 2A,E). The remaining 2 neurons were hyperpolarized (-7 and -4.5 mV) in response to 50 μM AICAR, while no cells were depolarized or hyperpolarized in response to 500 μM AICAR. Pretreatment with the AMPK inhibitor (compound C, 20uM) failed to alter the adiponectin-induced depolarization of *LepR*-positive *Pomc* neurons (58%; 6.0 ± 0.4 mV; resting membrane potential: -47.1 ± 2.7 mV; n $=$ 12; Figure 2B,E). These data suggest that adiponectin activates Pomc neurons that express leptin receptors independent of AMPK activity.

Interestingly, pretreatment with the PI3K antagonist (LY294002, 10 μM) abrogated the adiponectin-induced depolarization of *LepR*-positive *Pomc* neurons (100%; 0.5 ± 0.4 mV; resting membrane potential: -47.8 ± 3.3 mV; n $=$ 9; Figure 2C,E). Leptin depolarized *Pomc* neurons via a PI3K-dependent activation of *TrpC* channels [20,37]. Given the analogous effects of adiponectin observed in the current study, we hypothesized that adiponectin might also depolarize *Pomc* neurons via *TrpC* channels. Pretreatment with the non-selective *TrpC* channel inhibitors, 2-APB and SKF 96365, prevented the adiponectin-induced depolarization of *LepR*-positive *Pomc* neurons (2-APB: 100%; $+1.5 \pm 0.5$ mV; resting membrane potential: -46.1 ± 3.3 mV; n $=$ 7; SKF: $+1.7 \pm 0.7$ mV; resting membrane potential: -48.1 ± 4.3 mV; n $=$ 5; Figure 2D,E). Together, these data highlight a common mechanism in

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which adiponectin and leptin activate arcuate *Pomc* neurons (Figure 2E).

3.4. Adiponectin effects synaptic input organization of hypothalamic *Pomc* neurons

Pomc neurons receive and integrate afferent inputs from a variety of nuclei within and outside of the hypothalamus. In order to better describe the effects of adiponectin on the synaptic organization of arcuate *Pomc* neurons in the absence of voltage fluctuations, neurons were voltage-clamped at −75 mV (for excitatory postsynaptic currents) and −15 mV (for inhibitory postsynaptic currents). With a similar time course to the adiponectin-induced membrane depolarization, adiponectin (10 nM) decreased the frequency of spontaneous IPSCs in 10 of 14 *LepR*-positive *Pomc* neurons from 1.13 ± 0.36 Hz in normal ACSF to 0.4 ± 0.1 Hz in adiponectin (64.6% decrease from control; $p < 0.05$; paired t test; $n = 10$; Figure 3A–C). The amplitude of spontaneous IPSCs was decreased from 26.798 ± 3.91 pA in normal ACSF to 16.765 ± 1.99 pA in adiponectin (61.6% decrease from control; $p < 0.05$; paired t test; $n = 10$; Figure 3C). Oppositely, adiponectin (10 nM) resulted in a slight but not statistically significant decrease in spontaneous EPSCs (Figure 3D–F). The amplitude

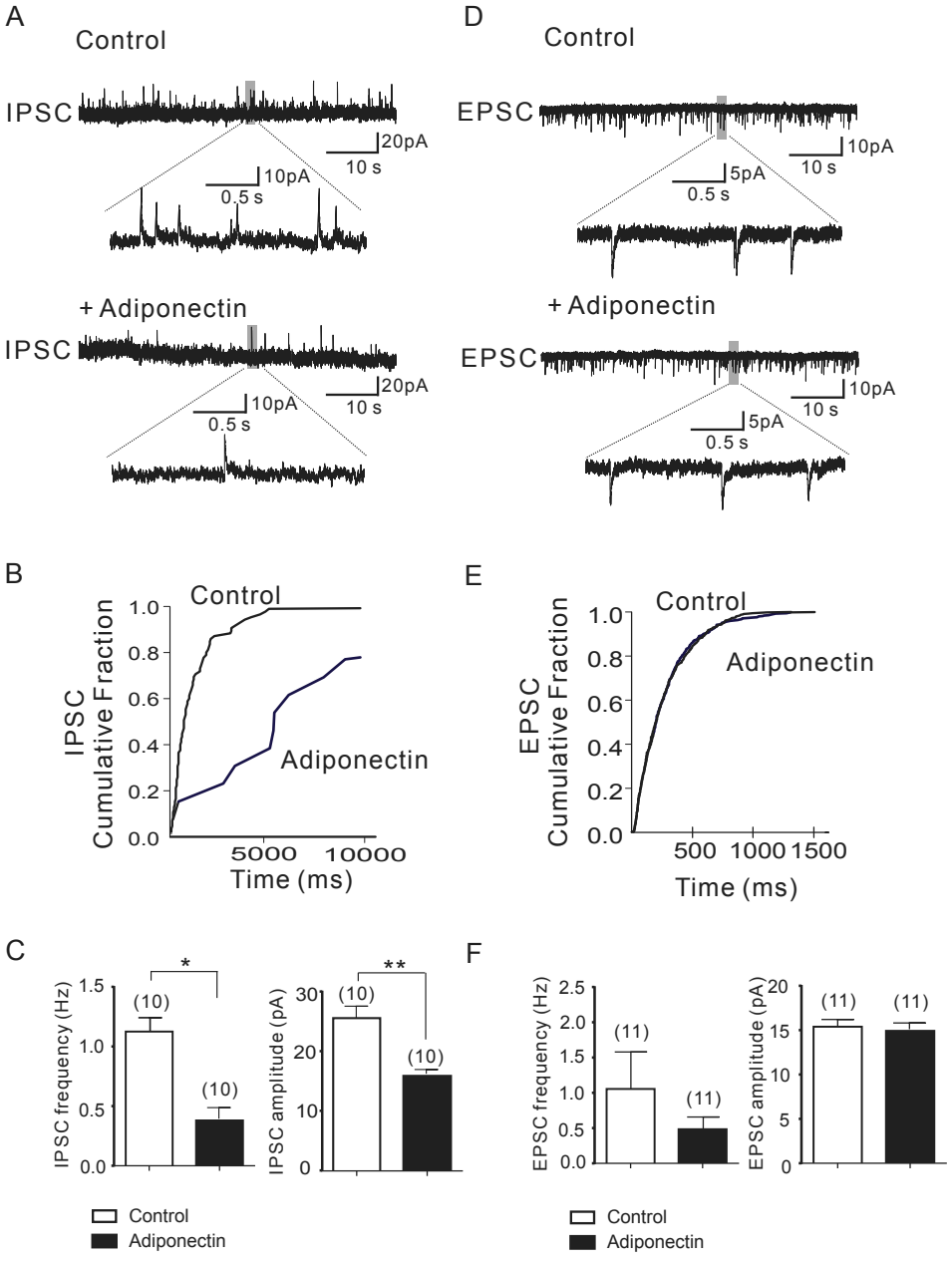

Figure 3: Adiponectin suppresses inhibitory synaptic input to arcuate *Pomc* neurons. (A) Spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron before and during adiponectin (10 nM; $V_m = -75$ mV). (B) Cumulative fraction plots shows a significant decrease in the frequency of sIPSCs in response to adiponectin (10 nM). (C) Plots indicating adiponectin-induced changes in sIPSC frequency and amplitude observed in the neuron population. (D) Spontaneous excitatory postsynaptic currents (sEPSCs) observed in *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron before and during adiponectin superfusion. (E) Cumulative fraction plot shows no significant change in sEPSCs frequency in response to adiponectin. (F) Frequency and amplitude of sEPSCs from *Pomc*-hrGFP::*LepR*-cre::tdtomato (green/red) neuron in response to adiponectin are summarized.

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of spontaneous EPSCs was also unaffected by adiponectin administration (15.39 ± 0.98 pA in normal ACSF; 14.92 ± 0.98 pA in adiponectin n = 11; Figure 3F).

3.5. Adiponectin inhibits the excitability of hypothalamic *NPY/AgRP* neurons

NPY/AgRP neurons, which commonly co-express the inhibitory neurotransmitter GABA, are immediately adjacent to arcuate *Pomc* neurons and are a known source for inhibitory synaptic input to arcuate *Pomc* neurons. Given that adiponectin selectively suppressed inhibitory synaptic activity to arcuate *Pomc* neurons, we examined whether adiponectin may suppress the excitability of *NPY/AgRP* neurons. *NPY/AgRP* neurons anatomically restricted to the arcuate nucleus of the hypothalamus were targeted from *NPY*-hrGFP mice [24]. Real-time visualization under fluorescence microscopy and Alexa Fluor350 hydrazide dye were used to dialyze neurons recorded (Figure 4A–D). Consistent with the decreased inhibitory synaptic activity to *Pomc* neurons, adiponectin perfusion hyperpolarized a subpopulation of *NPY* neurons (33.3%, 8 of 24 NPY neurons, −8.9 ± 1.3 mV; resting membrane potential: −44.3 ± 1.4 mV; n = 24; Figure 4E,H). Additionally, current–voltage relationships demonstrated a reversal potential of −80.3 mV ± 5.7 mV (n = 5; Figure 4F). Subsequent application of tolbutamide (200 uM) following adiponectin administration completely reversed the hyperpolarization of LepR-positive *NPY* neurons induced by adiponectin (n = 5). Furthermore, pretreatment of the slice with TTX (2 uM) failed to diminish the adiponectin-induced hyperpolarization of *NPY/AgRP* neurons (42.8%, −7.4 ± 0.4 mV resting membrane potential: −43.9 mV ± 2.3 mV; n = 7; Figure 4G,H), which indicates a direct membrane hyperpolarization independent of action-potential-mediated synaptic transmission. Overall, these data indicate that adiponectin hyperpolarizes *NPY* neurons via activation of an ATP-sensitive potassium conductance (K_{ATP}) independent of action-potential-mediated synaptic transmission (Figure 4H).

4. DISCUSSION

The predominant effect of adiponectin in *Pomc*-expressing neurons of the arcuate nucleus was excitatory, acting to depolarize neurons, decrease inhibitory synaptic inputs, and increase their responsiveness. Leptin potentiated these effects on *Pomc* cellular activity such that the leptin and adiponectin combined resulted in an enhanced

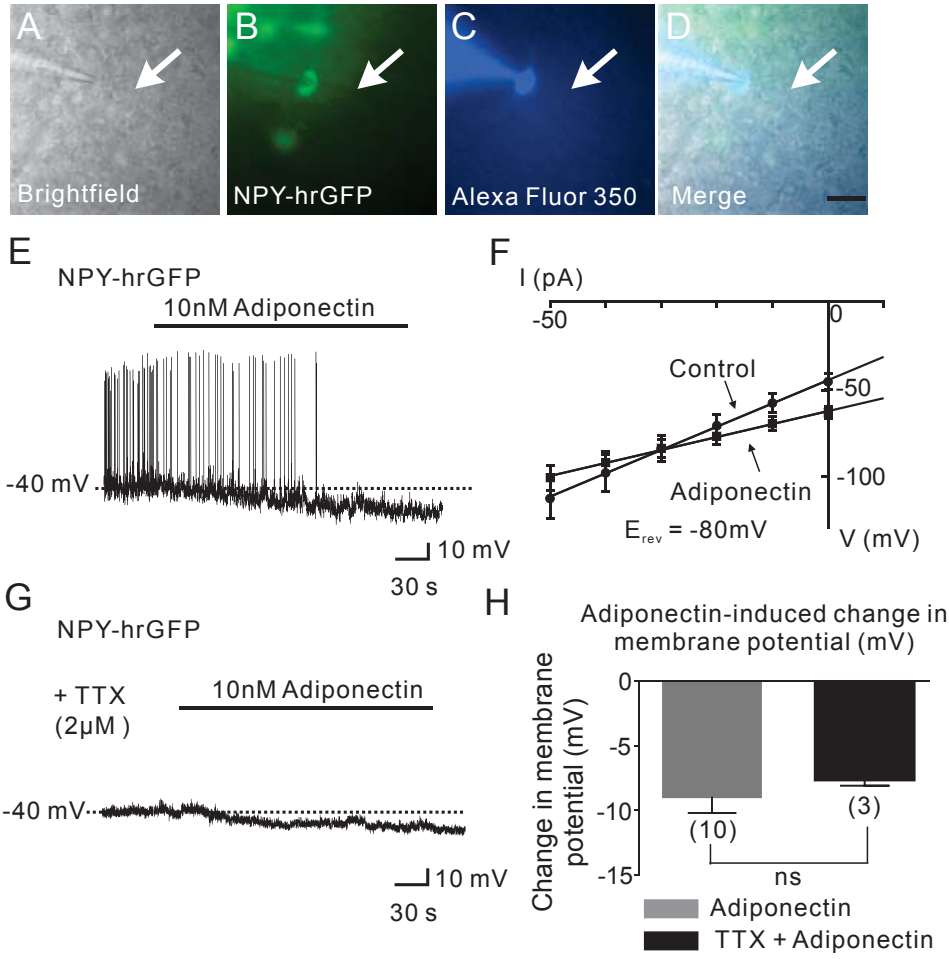

Figure 4: Adiponectin inhibits the excitability of hypothalamic *NPY* neurons. (A–D) Brightfield illumination (A) of *NPY*-hrGFP::*LepR*-cre::tdtomato neuron from NLT mice. (B) and (C) show the same neuron under FITC (hrGFP) and Alexa Fluor 350 illumination. Merged image of targeted *NPY* neuron is shown in (D). Arrow indicates the targeted cell. Scale bar = 50 μm. (E) Current-clamp record at resting membrane potential demonstrates an adiponectin-induced hyperpolarization of *NPY*-hrGFP neuron. (F) I–V relationship of *NPY*-hrGFP neuron in response to adiponectin. (G) Representative record shows that pretreatment with TTX does not abolish adiponectin-induced hyperpolarization of *NPY*-hrGFP (green) neuron. (H) Histogram illustrates the adiponectin-induced change of membrane potential of *NPY*-hrGFP (green) neurons with or without TTX. **p < 0.001. Error bars indicate SEM.

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depolarization when compared to either peptide alone. In contrast, the overall effect of adiponectin was inhibitory in adjacent *NPY/AgRP* neurons. These effects in combination with those from previous work support an adiponectin-dependent suppression of the melanocortin neural circuitry within the arcuate nucleus, which may promote a negative energy balance (summarized in Figure 5).

A novel observation in the current study is that adiponectin directly regulates the cellular activity of arcuate *Pomc* and *NPY/AgRP* neurons. Previous reports have suggested that adiponectin failed to alter the expression of various hypothalamic neuropeptides including *Pomc* and *NPY/AgRP* [13]. However, altered neuropeptide expression is a poor correlate of cellular activity. Similarly, adiponectin failed to induce expression of the immediate early gene c-fos in the arcuate nucleus suggesting that adiponectin failed to activate these neurons [13]. Although, c-fos has been associated with increased cellular excitability, this is at best an indirect measure of activity. In the current study, the adiponectin-induced activation of *Pomc* neurons occurred almost exclusively in leptin receptor expressing neurons. This is in agreement with the ability of adiponectin to potentiate the effects of leptin on thermogenesis, insulin sensitivity, body weight, fat and V_{02} utilization [13]. Moreover, adiponectin and leptin together enhanced UCP-1 mRNA expression in BAT [13]. Notably, melanocortin activity is an important regulator of the aforementioned alterations in energy balance and glucose homeostasis [38]. Accordingly, agouti (*Ay/a*) mice failed to respond to adiponectin or leptin, indicating the melanocortin pathway may be a common target [13]. We also found that adiponectin directly inhibited *NPY/AgRP* neurons further providing an indirect disinhibition (activation) of arcuate *Pomc* neurons. Although adiponectin suppressed *NPY/AgRP* neuronal activity, *Pomc* neurons that do not express leptin receptors failed to depolarize in response to adiponectin. This may suggest that the decreased GABA-ergic activity from *NPY/AgRP* neurons alone was not sufficient to modify the activity of these neurons. Alternatively, adiponectin may decrease inhibition of *NPY/AgRP* neurons to specific subsets of arcuate *Pomc* neurons as yet undefined. The current data may suggest that this subset would include *Pomc* neurons that express leptin receptors as *Pomc* neurons that do not express leptin receptors failed to respond to adiponectin. Although an intriguing hypothesis, this requires future investigation. Together, these data detail a cellular mechanism which supports an additive effect of leptin and adiponectin in melanocortin-expressing neurons.

To date, research on adiponectin receptors has focused primarily on the requirement of AMP-activated protein kinase (AMPK) signaling in peripheral tissues to regulate most aspects of metabolism [39]. Additional mechanisms widely studied include the effects of adiponectin and its receptors on sphingolipid metabolism, specifically with respect to lowering cellular ceramide levels [40]. However, there is a lesser-appreciated role for adiponectin's pleiotropic effects to activate pathways, which may either converge on or diverge from AMPK, including PI3K [40]. At the cellular level, adiponectin has been shown to activate Akt in endothelial cells, which can be blocked by PI3K inhibitors [41]. The adiponectin-induced activity of PI3K might also be intertwined with AMPK as inhibition of PI3K can abolish adiponectin-stimulated AMPK activation [42,43]. Unfortunately, little is known about the role of PI3K signaling in response to adiponectin in the central nervous system. However, the PI3K pathway in arcuate *Pomc* neurons has been demonstrated to be an important regulator of both thermogenesis and hepatic glucose production [20,44]. In particular, *Pomc* specific deficiency for the PI3K catalytic subunit p110alpha led to a hypometabolic phenotype contributing to increased weight gain and adiposity [20,44]. Alternatively, increased *PI3K* activity in *Pomc* neurons improved insulin sensitivity, whereas decreased *PI3K* signaling resulted in impaired glucose regulation [20,44]. Notably, these observations of PI3K signaling in *Pomc* neurons phenocopy the acute effect of adiponectin in the hypothalamus [13]. Accordingly, we found that the adiponectin-induced activation of arcuate *Pomc* neurons required PI3K signaling, independent of AMPK. Although currently unclear, these data support, at least in part, a cellular/molecular mechanism for adiponectin to alter energy balance and glucose metabolism via *PI3K* activity in melanocortin neurons.

Another salient finding is the temporal relationship of the electrophysiological responses observed. Similar to leptin, adiponectin activated arcuate *Pomc* neurons within minutes of application. These data suggest that adiponectin may parallel leptin activity in *Pomc* neurons; rapidly altering energy and glucose homeostasis via *Pomc* neuronal activity. In particular, previous work suggested that reduced *PI3K* activity in *Pomc* neurons alone was sufficient to block acute effect of leptin to activate *Pomc* neurons and reduce food intake up to 24 h after administration [20]. This supports a potential role of *PI3K*-dependent changes in *cellular* activity alone in *Pomc* neurons contributes to energy balance. Moreover, reports have suggested that chemogenetic and/or optogenetic stimulation of arcuate *Pomc* neurons reduces food intake within a range of a few hours to days after continual stimulation [45–47]. Given that arcuate *Pomc* cells have varying projection

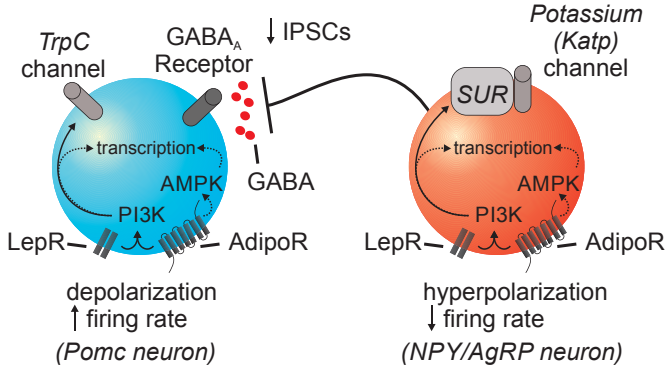

Figure 5: Schematic representation of the effects of adiponectin on hypothalamic *Pomc* and *NPY/AgRP* neurons. Adiponectin binds *AdipoRs* on *Pomc* neurons to depolarize *Pomc* neurons via PI3K mediated activation of *TrpC* channels independent of AMPK activity. Adiponectin also inhibits *NPY/AgRP* neurons via a PI3K-dependent activation of K_{atp} channels. This results in decreased inhibitory postsynaptic currents to neighboring *Pomc* neurons. Together, this proposed model may provide a molecular/cellular mechanism by which adiponectin decreases food intake and increases energy expenditure via integrating its effects on hypothalamic *Pomc* and *NPY/AgRP* neurons.

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patterns, which may separately influence feeding or autonomic behaviors [48—52], this highlights a need to understand which subpopulation of *Pomc* neurons are activated in order to better delineate the temporal regulation of metabolism. Together, while we cannot exclude other cellular processes; an adiponectin-induced activation of arcuate *Pomc* neurons may be sufficient to drive beneficial effects on energy balance and glucose homeostasis.

It should be noted that while adiponectin receptors are widely distributed in the CNS. A rise in CSF levels was detected after iv administration of adiponectin [13]. Peripheral administration of adiponectin stimulates cell signaling pathways in the hypothalamus of mice to improve metabolism [10,13]. Moreover, we have previously demonstrated the presence of the lower molecular weight complexes of adiponectin in the CSF at approximately 0.1% of serum concentration [53], which is within the concentration range of adiponectin used in the current study. However, others have failed to identify adiponectin BBB transport mechanisms and suggest that the brain endothelium may play an important role in relaying peripheral adiponectin signaling [54,55]. In the current study, we have not delineated whether peripheral or central administration of adiponectin is sufficient to initiate these responses. Rather, these data demonstrate that activation of adiponectin receptors (be it physiologically or pharmacologically) may positively regulate melanocortin activity.

5. CONCLUSIONS

In summary, leptin and adiponectin synergistically activate arcuate *Pomc* neurons via a PI3K-dependent activation of *TrpC* channels, independent of AMPK activity. These data may provide evidence for *PI3K* as a substrate for both leptin and adiponectin to regulate energy balance and glucose metabolism via melanocortin activity.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.08.007.

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