CRISPR/SaCas9 mutagenesis of stromal interaction molecule 1 in proopiomelanocortin neurons increases glutamatergic excitability and protects against diet-induced obesity

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ABSTRACT

Objective: Proopiomelanocortin (POMC) neurons are the key anorexigenic hypothalamic neuron for integrating metabolic cues to generate the appropriate output for maintaining energy homeostasis and express the requisite channels as a perfect synaptic integrator in this role. Similar to the metabolic hormones leptin and insulin, glutamate also excites POMC neurons via group I metabotropic glutamate receptors (mGluR1 and 5, mGluR1/5) that activate Transient Receptor Potential Canonical (TRPC 5) Channels to cause depolarization. A key modulator of TRPC 5 channel activity is stromal interaction molecule 1 (STIM1), which is involved in recruitment of TRPC 5 channels from receptor-operated to store-operated calcium entry following depletion of calcium from the endoplasmic reticulum.

Methods: We used a single adeno-associated viral (AAV) vector containing a recombinase-dependent Staphylococcus aureus Cas9 (SaCas) and a single guide RNA (sgRNA) to mutate Stim1 in POMCCre neurons in male mice, verified by qPCR of Stim1 mRNA expression in single POMC neurons. Whole-cell patch clamp experiments were conducted to validate the effects of Stim1 mutagenesis. Body weight and food intake were measured in male mice to assess disruptions in energy balance.

Results: Reduced Stim1 expression augmented the efficacy of the mGluR1/5 agonist 3, 5-Dihydroxyphenylglycine (DHPG) to depolarize POMC neurons via a Gαq-coupled signaling pathway, which is an essential part of excitatory glutamatergic input in regulating energy homeostasis. The TRPC 5 channel blockers HC070 and Pico145 antagonized the excitatory effects of DHPG. As proof of principle, mutagenesis of Stim1 in POMC neurons reduced food intake, attenuated weight gain, reduced body fat and fat pad mass in mice fed a high fat diet.

Conclusions: Using CRISPR technology we have uncovered a critical role of STIM1 in modulating glutamatergic activation of TRPC 5 channels in POMC neurons, which ultimately is important for maintaining energy balance.

Keywords CRISPR/SaCas9; POMC; STIM1; TRPC 5 channel; Glutamate; High fat diet

1. INTRODUCTION

Mammalian TRPC channels can be activated by G protein-coupled receptors and receptor tyrosine kinases [1,2] and are one of the major targets for group I metabotropic glutamate receptors (mGluR1/5)-mediated signaling in CNS neurons [3—6]. For example, TRPC 5 channels are highly expressed in substantia nigra dopamine neurons, and mGluR1/5 agonists such as DHPG ((S)-3,5-Dihydroxyphenylglycine) induce an inward current that exhibits a double-rectifying current—voltage plot [3]. Similarly, DHPG depolarizes POMC neurons, and high frequency stimulation of arcuate nucleus kisspeptin (Kiss1R) neurons releases enough glutamate to “spill over” and activate extrasynaptic metabotropic glutamate (mGluR1/5) receptors [7]. mGluR1/5 are Gαq-coupled to phospholipase C (PLC) activation, which leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3) that mediate further downstream actions. One of the targets is the TRPC 5 channel, which is a cation selective channel and can associate with Orai calcium channels to form

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calcium release-activated calcium (CRAC) channels [8]. TRPC 5 channels are highly permeable to calcium (P_{Ca/PNa} = 9.1) [9], and a unique feature of TRPC 5 (and TRPC 4) channels is that they are potentiated by micromolar concentrations of lanthanum (La^{3+}) [10], which we have exploited to characterize TRPC 5 signaling in POMC neurons [11,12]. TRPC channels can function as receptor-operated channels or as store-operated channels depending on their association with the endoplasmic reticulum (ER) protein stromal interaction molecule 1 (STIM1) [8]. STIM1 is localized to the ER membrane of cells and its N-terminal domain contains an EF-hand that protrudes into the lumen of the ER to sense changes in ER Ca^{2+} concentrations [13]. Upon depletion of ER Ca^{2+}, STIM1 undergoes a conformational change, oligomerizes and then interacts with plasma membrane TRPC channels [13,14].

Previously, we discovered that the efficacy of insulin to activate TRPC 5 channels and depolarize POMC neurons was significantly reduced in diet-induced obesity male but not female mice [15]. The insulin response in POMC neurons was abrogated in ovariectomized, DIO females but restored with estradiol (E2) replacement—i.e., E2 protected POMC neurons against insulin resistance. Also, E2 down-regulated Stim1 mRNA, which rendered POMC neurons more excitable and more responsive to insulin-mediated TRPC 5 channel activation [15]. In addition, we found that the insulin-induced TRPC 5 current in POMC neurons in ovariectomized females was enhanced in the presence of a store-operated Ca^{2+} channel inhibitor. We have also observed that longer term E2 treatment down-regulates Stim1 mRNA expression by ~2 fold in the arcuate nucleus of female guinea pigs, indicating that this E2-mediated mechanism for increasing TRPC 5 channel coupling to insulin receptors may be conserved among mammals. Therefore, we hypothesized that under normal physiological conditions, TRPC 5 channels function similarly to other plasma membrane calcium channels in POMC neurons but can also be coupled to plasma membrane receptors (insR, Lrb, mGluR1/5) [7,11,12]. However, in cellular stressed states such as with obesity TRPC 5 channels associate with STIM1 and are coupled to Ca^{2+} store depletion from the endoplasmic reticulum. E2 appears to play a protective role by downregulating Stim1 mRNA expression. To test this hypothesis, we sought to selectively delete Stim1 expression in POMC neurons by crossing Pomc^{Cre} mouse with Stim1^floX/m^ mice, thereby producing conditional knockout of Stim1 in POMC neurons. We had previously used this strategy to delete Stim1 from Kiss1^{REH} neurons [16]. However after extensive breeding over some twenty litters, we were unable to generate viable Pomc^{Cre}:Stim1^{floX}/m offspring, even though we were able to produce Kiss1^{Cre}:Stim1^{floX}/m offspring that survived well into adulthood [16]. Therefore, we took advantage of a newly developed CRISPR technology [17,18] in which we utilized a single adeno-associated viral (AAV) vector containing a recombinase-dependent Staphylococcus aureus Cas9 (SaCas) and a single guide RNA (sgRNA) to selectively mutategen Stim1 in POMC neurons in male mice and measure changes in both cellular excitability and the whole animal physiological responses to high fat diet.

2. MATERIALS AND METHODS

2.1. Animals and treatments

All the animal procedures described in this study were performed in accordance with institutional guidelines based on National Institutes of Health standards and approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University or the University of Washington, Seattle.

2.2. Mice

Pomc^{Cre} (RRID:IMSR_JAX:005965, strain of origin: FVB/N) [19], Pomc^{cgrp} (RRID: I M S R _ J A X : 0 0 9 5 9 3 , s t r a i n o f o r i g i n : C 5 7 B L / 6 J ) [ 2 0 ] , and Stim1^{+/-} (RRID: IMSR_JAX: 023,350, strain of origin: B6. Cg-Thy1a^{21}) transgenic mice were selectively bred at OHSU. D A Y ^ C R A C S o (RRID: IMSR_JAX: 006660, strain of origin: not specified) [22] transgenic mice were selectively bred at University of Washington. All animals were maintained under controlled temperature and photoperiod (lights on at 0600 h and off at 1800h) and given free access to food and water. We utilized a well-established, diet-induced obesity mouse model in order to do cellular studies on POMC neurons [23,24]. Pomc^{Cre} mice were put on a high fat diet (HFD, 45% kcal from fat, Research Diets, NJ, D12451) starting at 10 weeks of age for 8 weeks in order to induce diet-induced obesity (DIO). A control group of mice received normal grain-based chow (5L0D; Lab Diets). After 7–8 weeks, we prepared coronal slices from both groups of mice and did whole-cell patch recordings from Pomc^{Cre} (POMC) neurons.

2.3. Generation and validation of AAV1-FLEX-SaCas9-U6-sgStim1

The sgRNA for targeted mutagenesis of Stim1 exon 3 was designed as previously described [17]. Oligos (Sigma) for the sgRNA were cloned into pAAV-FLEX-SaCas9-U6-sgRNA (Addgene 124,844, Stim1 forward: CACCGAAAAACAAGACATCTTCCATTTG; Stim1 reverse: AAAGACATGGAAGGTCATCGTATTTT. The control sgRNA oligos contained a three base pair substitution located at the seed region upstream of the PAM. Stim1TTA control forward: CACCGAAAAACATAGCCCTCCATTTA; Stim1TTA control reverse: AACAATATTGGAAAAAGTCTATC. For targeted deep sequencing of the Stim1 locus: AAV1-FLEX-SaCas9-U6-sgStim1 and AAV1-FLEX-EGFP-KASH were co-injected into the ventral tegmental area (VTA) of D A Y ^ C R A C mice. Four weeks following surgery, tissue punches of the ventral midbrain from 3 mice were pooled and nuclei were isolated by FACS as described previously [17]. For nuclear isolation, brain tissue was homogenized in buffer containing the following (in mM): 320 Sucrose (sterile filtered), 5 CaCl_{2} (sterile filtered), 3 Mg(Ac)_{2} (sterile filtered), 10 Tris pH 7.8 (sterile filtered), 0.1 EDTA pH 8 (sterile filtered), 0.1% NP40, 0.1 Protease Inhibitor Cocktail (PIC, Sigma), 1 β-mercaptoethanol. Optiprep density gradient medium (5 ml of 50%, Sigma) containing (in mM): 5 CaCl_{2} (sterile filtered), 3 Mg(Ac)_{2} (sterile filtered), 10 Tris pH 7.8 (sterile filtered), 0.1 PIC, 1 β-mercaptoethanol was added to the homogenate and mixed by inversion. The mixture was loaded onto 10 ml of 29% iso-osmolar Optiprep solution and centrifuged at 7500 RPM for 30 min at 4 °C. The pellet was suspended in sterile 1xPBS and nuclei were sorted using a BD AriaFACS III, followed by whole genome amplification (WGA) using REPLI-g Advanced DNA Single Cell kit (Qiagen) according to manufacturer’s instructions. Two rounds of targeted PCR were performed using the following primer sets. Primer set 1 forward: TAGTG- CATTGGAAACTGTG; primer set 1 reverse: CCATGCTCCCTTACGGAC; primer set 2 forward: CTGGAGACATGTAAGCT; primer set 2 reverse: CTATCCATCCCTGTTTC. A 260 base pair amplicon from primer set 2 was gel extracted and submitted for deep sequencing (Amplicon-EZ, GeneWiz).

2.4. AAV delivery to Pomc^{Cre} mice

To visualize the quality and location of the injection/infection, the CRISPR/SaCas9 vector was spiked with a high titer virus encoding mCherry or YFP. The resulting mixture allowed a single injection of both viruses at a similar titer. Co-injected viruses were always the same serotype (AAV1) so as not to affect the transduction efficiency [17]. Three to eight weeks prior to each experiment, the Pomc^{Cre} mice
section for specific s on the sgRNA design) or GCaMP6s (AAV9-SynFlex-GCaMP6s-WPRE-SV40; Addgene, #100845-AAV9). Using asceptic techniques, anesthetized mice (1.5% isoflurane/O2) received a skin incision to expose the surface of the skull. The glass pipette (Drummond Scientific #3-000-203-GX) with a beveled tip (diameter = 45 μm) was filled with mineral oil, loaded with an aliquot of AAV using a Nanoject II (Drummond Scientific). ARH injection coordinates were anteriorposterior (AP): −1.20 mm, mediolateral (ML): ±0.30 mm, dorsoventral (DL): −5.80 mm (surface of brain z = 0 mm); 500 nl of the AAV (2.0 × 10^12 particles/ml) was injected (100 nl/min) into each position, and the pipette left in place for 10 min post-injection, then slowly retracted from the brain. The skin incision was closed using Vetbond (3 M) and each mouse received analgesia (Rimadyl, 4–5 mg/kg, s. c.).

2.5. Calcium imaging

For calcium imaging, coronal arcuate slices (250 μm) were prepared from intact PomcCre males which had received bilateral ARH injections of a Cre-dependent adeno-associated viral (AAV; serotype 1) vector encoding yellow fluorescent protein, YFP (AAV1-EF1α-YFP), or mCherry mCh (AAV1-EF1α-mCh) either alone or co-injected with an AAV1 designed to encode SaCas9 and a single-guide RNA (sgRNA) (See the SaCas9 section for specifics on the sgRNA design) or GCaMP6s (AAV9-SynFlex-GCaMP6s-WPRE-SV40; Addgene, #100845-AAV9). Using asceptic techniques, anesthetized mice (1.5% isoflurane/O2) received a skin incision to expose the surface of the skull. The glass pipette (Drummond Scientific #3-000-203-GX) with a beveled tip (diameter = 45 μm) was filled with mineral oil, loaded with an aliquot of AAV using a Nanoject II (Drummond Scientific). ARH injection coordinates were anteriorposterior (AP): −1.20 mm, mediolateral (ML): ±0.30 mm, dorsoventral (DL): −5.80 mm (surface of brain z = 0 mm); 500 nl of the AAV (2.0 × 10^12 particles/ml) was injected (100 nl/min) into each position, and the pipette left in place for 10 min post-injection, then slowly retracted from the brain. The skin incision was closed using Vetbond (3 M) and each mouse received analgesia (Rimadyl, 4–5 mg/kg, s. c.).

2.5. Calcium imaging

For calcium imaging, coronal arcuate slices (250 μm) were prepared from intact PomcCre males which had received bilateral ARH injections of a Cre-dependent adeno-associated viral (AAV; serotype 1) vector encoding GCaMP6s (RRID:Addgene_100,842) two to three weeks prior to the experiments as previously described [16]. The brain slices were analyzed using p-Clamp software (Molecular Devices, Foster City, CA). The liquid junction potential was corrected for all data analysis. I–V relationships of the drug-induced (i.e., DHPG) currents were constructed by voltage ramps from −100 to +10 mV from a holding potential of −60 mV.

2.7. Electrophysiological solutions/drugs

A standard artificial cerebrospinal fluid was used [11]. All drugs were purchased from Tocris Bioscience unless otherwise specified. TTX was purchased from Alomone Labs (1 mM), l-glutamic acid (AP5; 50 mM), 6-cyano-7-nitroquinolxaline-2,3-dione (CNQX; 10 mM) and Selective group I mGlur agonist, (S)-3,5-Dihydroxyphenylglycine (DHPG, 50 mM) were dissolved in H2O. Picrotoxin (100 mM), TRPC4/5 antagonist HC070 and TRPC1/4/5 antagonist Pico145 (from MedChemExpress, 10 mM), and Calcium release-activated Ca^2+ (CRAC) channel Inhibitor GSK7975A (10 mM) from AOBIOUS Inc. (Gloucester, MA) were prepared in dimethylsulfoxide (DMSO). Aliquots of the stock solutions were stored as appropriate until needed.

2.8. Glucose tolerance test (GTT)

Mice were housed individually, and body weights were measured once a week. The evening prior to each glucose tolerance test (GTT), all mice were fasted for 15-h and then injected intraperitoneally with glucose (1 mg/g lean mass as determined by MRI) [25] in sterile PBS and blood glucose levels were measured 15, 30, 60, 90, and 120 min after injection.

2.9. Cell harvesting of dispersed PomcEGFP and PomcCre neurons labeled with YFP and quantitative real-time PCR (qPCR)

Cell harvesting and qPCR was conducted as previously described [26]. The ARH was microdissected from basal hypothalamic coronal slices transferred to an auxiliary chamber in which they were kept at room temperature (25 °C) in artificial CSF (aCSF) consisting of the following (in mM): 124 NaCl, 5 KCl, 2.6 NaH2PO4, 2 MgSO4, 2 CaCl2, 26 NaHCO3, 10 HEPES, 10 glucose, pH 7.4, until recording (recovery for 2 h). A single slice was transferred to the recording chamber at a time and was kept viable by continually perfusing with warm (35 °C), oxygenated aCSF at 1.25 ml/min. Whole-cell patch recordings were made from PomcEGFP neurons using an Olympus BX51 W1 fixed stage scope out-fitted with epifluorescence and infrared-differential interference contrast (IR-DIC) video microscopy. Patch pipettes (A-M Systems; 1.5 μm outer diameter borosilicate glass) were pulled on a Brown/Fleming puller (Sutter Instrument, model P-97) and filled with the following solution: 128 mM potassium glutconate, 10 mM NaCl, 1 mM MgCl2, 11 mM EGTA, 10 mM HEPES, 3 mM ATP, and 0.25 mM GTP adjusted to pH 7.3 with KOH; 295 mOsm. Pipette resistances ranged from 3.5 to 4 MΩ. In whole-cell configuration, access resistance was less than 30 MΩ; the access resistance was 80% compensated. The input resistance was calculated by measuring the slope of the I–V relationship curve between −70 and −50 mV. Standard whole-cell patch recording procedures and pharmacological testing were performed as previously described [11]. Electrophysiological signals were digitized with a Digidata 1322 A (Axon Instruments) and the data were analyzed using p-Clamp software (Molecular Devices, Foster City, CA).
obtained from intact male PomcEGFP mice (n = 3) and PomcCre mice (n = 4 animals/group). The dispersed cells were visualized, patched, and then harvested (10 cells/tube) as described previously [26]. Briefly, the tissue was incubated in papain (7 mg/ml in oxygenated acSF) for 50 min at 37C and washed 4 times in low Ca2+ acSF and two times in aCSF. Gentle trituration with Pasteur pipettes were used to disperse the neurons onto a glass bottom dish. Oxygenated aCSF circulated into the plate keeping the cells clear of debris. Only healthy cells with processes and a smooth cell membrane were harvested. The cells were harvested using the XenoWorks Microinjector System (Sutter Instruments, Navato, CA), which provided negative pressure in the pipette and fine control to draw the cell up into the pipette. Cells were harvested as pools of 10 individual cells/tube.

Primers for the genes that encode for stromal-interaction molecule 1 (Stim1), Stim2 and β-actin were designed using Clone Manager software (Sci Ed Software) to cross at least one intron-exon boundary and optimized as previously described using Power Sybr Green method [26,27]. We have published the primers for β-actin [7] and Stim1 (Stim1 primers span the sgRNA and PAM) and Stim2 [16]. Controls included neuronal pools reacted without reverse transcriptase (RT), hypothalamic RNA reacted with RT and without RT, as well as water blanks. Primers for qPCR were further tested for efficiency (E = 10−1/m - 1) [26,28,29]. The results were as follows: β-actin, m = -3.465, r² = 0.95, efficiency = 95%; Stim1; m = -3.311, r² = 0.98, efficiency = 100%. Stim2; m = -3.439, r² = 0.99, efficiency = 95%. qPCR was performed on a Quansstudio 7 Flex Real-Time PCR System (Life Technologies) using Power Sybrgreen (Life Technologies) mastermix according to established protocols [26]. The comparative ∆∆CT method [28,29] was used to determine values from duplicate samples of 4 μl for the target genes, Stim1 and Stim2 and 2 μl for the reference gene β-actin in a 20 μl reaction volume containing 1× Power SYBR Green PCR Master Mix (Applied Biosystems) and 0.5 μM forward and reverse primers. The relative linear quantity was determined using the 2−ΔΔCT equation [26]. In order to determine the relative expression levels of target genes in POMC-YFP neurons obtained from controls as compared to mutated animals, the mean Δ CT for the target genes from the control samples were used as the calibrator. For the POMC-EGFP neurons obtained from intact male animals a comparison was made between Stim1 expression and Stim2 expression and the mean Δ CT from Stim1 was used as the calibrator. The data were expressed as n-fold change in gene expression normalized to the reference gene β-actin and relative to the calibrator and the mean and standard error of the mean were calculated and used for statistical analysis.

2.10. Experimental design and statistical analysis
In visualized whole-cell patch recording experiments, only one recording was made per slice, and a maximum of 3 recordings were made from each PomcCre mouse. For cell harvesting of dispersed POMC-YFP and POMC-EGFP neurons and quantitative real-time PCR (qPCR) measurements, 10 cells per pool and 4–5 pools from each animal were used. For the mouse body composition, food intake and face the deleterious developmental impact of Stim1 loss of function in PomcCre cells, we selectively mutated Stim1 using a single viral vector CRISPR/SaCas9 approach [17] that allows for conditional mutagenesis in the adult nervous system. CRISPR/Cas9 is recognized as an efficient means of generating insertion/deletion (indel) mutations to cause a loss of function in targeted genes [33–35] with minimal off-target effects [36]. Importantly, there is no unexpected CRISPR/Cas9 off-target activity revealed by trio sequencing of gene-edited mice [36], and trio deep-sequencing does not reveal unexpected off-target and on-target mutations in Cas9-edited rhesus monkeys [37]. To inactivate Stim1, we generated a guide targeting exon 3. Because of the small size of the ARH and the relatively low number of POMC neurons, we confirmed the efficacy of Cas9 mutagenesis of Stim1 in midbrain dopamine neurons as previously described [17]. Briefly, the DATCre mice were co-injected with AAV1-FLEX-SaCas9- U6-sgStim1 and AAV1-FLEX-EGFP-KASH, and tissue was harvested four weeks following surgery. EGFP-positive and -negative nuclei were isolated by FACs and whole genome amplification (WGA) was performed followed by targeted deep sequencing of a PCR amplicon containing the targeted region of Stim1 (Figure 1A–C). Cas9 generated numerous insertions and deletions (indels) centered at 3 base pairs upstream of the protospeacer adjacent motif (PAM) (Figure 1A and C-E).

Based on the promising results of Stim1 editing by the SaCas9/sgRNA vector in the DATCre mice, a cohort of PomcCre mice was given bilateral stereotaxic injections in the ARH of either AAV1-FLEX-SaCas9-U6- sgStim1 or a control virus containing the Stim1 guide with three base pairs in the mutated seed region (SaCas9-control) as previously described [17]. An additional Cre-dependent virus with the same serotype (AAV1) that drove the expression of a fluorophore (YFP or mCherry) was co-administered to visualize the injection quality and facilitate cell harvesting (Figure 1F–G). To determine the impact of mutagenesis on Stim1 expression in ARH PomcCre neurons, we performed single cell qPCR to assess nonsense mediated mRNA decay which has been described following targeted CRISPR/SaCas9 mutagenesis [38]. Brain slices were prepared, and cells were harvested as previously described [39] and analyzed with qPCR. In cerebellar Purkinje neurons, Stim1 is more abundant than STIM2 [30], while in hippocampal [40] and cortical neurons [41] STIM2 levels exceed those of Stim1. In ARH- PomcCre neurons, we found that Stim1 mRNA is 2.9-fold greater than Stim2 mRNA (Figure 1H) and CRISPR/Cas9 mutagenesis significantly reduced Stim1 mRNA levels in these cells (Figure 1I).
3.2 Stim1 mutagenesis decreases the excitability of PomcCre neurons

Recently, we investigated the details of the spike properties in Kiss1ARH neurons with deletion of Stim1 and found that there were no differences between Kiss1ARH neurons from control Kiss1Cre and Stim1ko mice in the firing frequency—current relationship [16]. Nevertheless, in cerebellar Purkinje neurons from Stim1ko mice, the firing frequency versus injected current relationship shows a reduced excitability [31]. Based on these observations, we asked whether mutagenesis of Stim1 with the CRISPR/SaCas9 strategy would alter the activity of these cells. To investigate whether STIM1 modulates the activity of PomcCre neurons, we bilaterally injected either AAV1-FLEX-SaCas9-U6-sgStim1 or a control virus with AAV1-Ef1a-DIO-ChR2:mCherry into the ARH of PomcCre male mice. Initially, whole-cell patch recording in POMC neurons from PomcCre or Stim1 mutagenesis (Stim1Mut) male mice revealed that there was no difference in the resting membrane potential

Figure 1: Analysis of targeted Stim1 mutagenesis in DATCre mice and POMC neurons from PomcCre mice. A, schematic of a sagittal section showing the viral injections of AAV1-FLEX-SaCas9-U6-sg Stim1 and control virus into the VTA of adult male DATCre mice. B, design of AAV1-FLEX-SaCas9-U6-sgStim1; the PAM is underlined. C, top 10 mutations at the cut site (black arrow) with the percent of total reads for which they occur on the left. Base changes: bolded. Insertions: underlined. Deletions: marked with a “-” (dash). D, frequency distribution of insertions and deletions in Stim1 from GFP+ nuclei. E, percent of wild-type, deletions, insertions, and base changes as percent of total reads for Stim1 in GFP+. F, schematic of a coronal section showing the bilateral viral injections in the ARH with AAV1-DIO-YFP. G, photomicrograph showing coronal section confirming targeted bilateral injections of AAV1-FLEX-SaCas9-U6-sgStim1 and control virus into the arcuate of adult male PomcCre mice. Scale, 200 μm. H, quantitative PCR assay measuring Stim1 and Stim2 in POMC neuronal pools (n = 3 animals, 10 cells per pool, 5 pools/animal) from Pomc-EGFP male control mice. Bar graphs represent mean ± SEM (Unpaired t-test for the left, t(4) = 5.092, **p = 0.0070). n = animal number. I, quantitative PCR assay measuring Stim1 in POMC neuronal pools (n = 4 animals, 10 cells per pool, 4 pools/animal) using STIM1(135 bp) primers that span the sgRNA and PAM from PomcCre control and Stim1Mut male mice. Bar graphs represent mean ± SEM (Unpaired t-test, t(6) = 5.275, **p = 0.0019).
membrane capacitance (Figure 2B) (Cm: 6 mF/C6).

Cells, but the (Figure 2A) (RMP: Figure 2: Electrophysiological properties of POMC neurons from PomcCre and Stim1Mut male mice. A. Resting membrane potential (RMP), B. Cell capacitance (Cm), C. Input resistance (Rin). Unpaired t-test, t(49) = 2.903, **p = 0.0055, PomcCre vs Stim1Mut, n = 25; Stim1Mut, n = 26. D. Rheobase. E. firing frequency. There were significant differences in the evoked firing rate between PomcCre and Stim1Mut groups (two-way ANOVA: main effect of treatment, F1,49 = 4.464, p = 0.0452, main effect of current, F8,192 = 108.7, p < 0.0001, and interaction, F8,192 = 3.223, p = 0.0018; PomcCre, n = 13, Stim1Mut, n = 13; post hoc Bonferroni test, *p < 0.05 and **p < 0.01). F. overshoot. Unpaired t-test, t(49) = 3.127, **p = 0.0039, PomcCre vs Stim1Mut, n = 18; Stim1Mut, n = 14, G, full width at half maximum (FWHM). Unpaired t-test, t(49) = 2.232, *p = 0.0322, PomcCre, n = 18; Stim1Mut, n = 14. H, Peak to afterhyperpolarization (AHP) amplitude. Unpaired t-test, t(25) = 2.356, *p = 0.0266, PomcCre vs Stim1Mut, n = 17; Stim1Mut, n = 10. I, threshold. J, AHP amplitude. K, AHP duration. L, Phase plots of elicited action potentials.

(Figure 2A) (RMP: PomcCre, 67 ± 1.6 mV, n = 27 vs Stim1Mut, 64 ± 1.5 mV, n = 32. Unpaired t-test, t(57) = 0.9495, p = 0.3464) or membrane capacitance (Figure 2B) (Cm: PomcCre, 22.1 ± 1.4 pF, n = 25 vs Stim1Mut, 21.8 ± 1.1 pF, n = 27. Unpaired t-test, t(50) = 0.1322, p = 0.8953). However, there was a significant difference in the membrane input resistance (Figure 2C) (Rin: PomcCre: 1.6 ± 0.2 GΩ, n = 25, vs Stim1Mut: 0.9 ± 0.0 GΩ, n = 26, Unpaired t-test, t(49) = 2.903, p = 0.0055), which has also been reported with Stim1 knockout in both cerebellar Purkinje neurons [31] and arcuate Kiss1 neurons [16]. To investigate spiking properties, we injected step currents into POMC neurons in current-clamp recordings, and we also measured the rheobase in the presence of synaptic blockers (CNQX, AP5, and picrotoxin) and the frequency–current relationship. The rheobase in POMC control neurons was not different from Stim1Mut cells, but the firing frequency versus injected current curve showed a reduced excitability in POMC neurons from Stim1Mut mice (Figure 2D–E). Through analysis of the characteristics of single evoked action potentials, we observed that mutagenesis of Stim1 reduced overshoot and peak to AHP amplitude, but increased full width at half maximum (FWHM) (Figure 2F–H) without affecting threshold, AHP amplitude and AHP duration (Figure 2I–K). However, there was a pronounced decrease in the maximum rise and decay velocity for the generation of an action potential (Figure 2L), which may be driven, in part, by calcium-activated potassium conductances similar to what has been observed in cerebellar Purkinje neurons [31].

3.3. The store-operated Ca2+ channel inhibitor GSK7975A augments the DHPG-induced current in POMC neurons

Although there appeared to be a decrease in endogenous (autonomic) cell excitability, we hypothesized that POMC neurons may be more coupled to exogenous synaptic input with Stim1 mutagenesis. Therefore, we explored glutamatergic input and specifically the effects of mGlurR1/5 agonist activity. Group I mGlurRs (mGlur 1/5) couple to Calcium (Ca2+) is of critical importance to neurons as it participates in the transmission of the depolarizing signal and contributes to synaptic activity [42]. We first measured the effects of the mGlurR1/5 agonist DHPG on GCaMP6s-expressing PomcCre neurons at different concentrations in arcuate slices from PomcCre mice. Different concentrations of DHPG (0.5, 5, and 50 µM) were constantly perfused over naïve slices while fluorescence was monitored. DHPG dose-dependently induced an increase in [Ca2+] with maximal dose at 50 µM (Figure 3A,B). At 50 µM DHPG, nearly every POMC neuron displayed a rapid and significant increase in fluorescence. The responsibility of POMC neurons to DHPG did not seem to differ based on location, neither medial to lateral within a slice nor rostral caudal between slices. Therefore, we used the mGlur 1/5 agonist DHPG at 50 µM to explore its action in POMC neurons, and found that DHPG induced an inward current in synaptically-isolated POMC neurons in the presence of TTX to block voltage-gated Na+ channels (Figure 3C). We antagonized the effects of DHPG with the selective TRPC4,5 channel blocker.
Our previous findings that deletion of *Stim1* in hypothalamic arcuate nucleus Kiss1 neurons can augment the TRPC 5 channel conductance [16], we asked whether STIM1 can modulate mGluR1/5-mediated excitability through Gq-coupling to TRPC 5 channel activation [16]. Glutamatergic excitation of POMC neurons plays a critical role in regulating energy homeostasis, and the Gq-coupled mGluR1/5 are a critical part of the signaling pathway [7]. *Stim1* mRNA is highly expressed in POMC neurons and in the presence of a store operated Ca2+ channel inhibitor GSK7975A (10 μM) (F), 0.1% v/v DMSO vehicle control had no effect (data not shown). DHPG-induced inward currents were recorded in arcuate YFP labeled neurons (Vhold = −30 mV) from *Pomc*+/− mice. A, the I–V relationship before and during the peak response from the same cell in F indicated that the reversal potential of the nonselective cation current was ~ −30 mV. H, summary of the effects of GSK7975A, HC070 and Pic145 on DHPG-induced current (one-way ANOVA, effect of treatment, F(3, 32) = 16.97, p < 0.0001; Bonferroni’s multiple comparisons test a,b,c, p < 0.05; d, ns.). Scatter plots represent mean ± SEM. n = 11 control, n = 6 HC070, n = 5 pic145 and n = 14 GSK7975A treated neurons.

HC070 [43]. HC070 suppressed the DHPG-induced inward current by 70% in POMC neurons similar to the extent seen with another more selective TRPC 5 channel blocker Pic145 ([Figure 3D, E and H]) suggesting that DHPG mainly activates TRPC 5 channels. Based on our previous findings that deletion of *Stim1* in hypothalamic arcuate nucleus Kiss1 neurons augments the DHPG-induced current in POMC neurons by glutamatergic inputs in mice on a HFD. To test the hypotheses, we measured the DHPG-induced current and found that in the presence of TTX, DHPG induced an inward current in POMC neurons (~ 30 mV), which is similar to the DHPG-induced current without treatment of GSK7975A ([Figure 3G]), indicating that the same cationic current is driving the increased conductance.

### 3.4 *Stim1* mutagenesis augments the DHPG-induced current in POMC neurons from mice on a HFD

Our previous findings show that deletion of *Stim1* in hypothalamic arcuate nucleus Kiss1 neurons protects against diet-induced obesity [16]. In Kiss1<sup>cre</sup> neurons, conditionally deleted *Stim1* augments tachykinin 3 receptor agonist senktide-induced Kiss1<sup>cre</sup> neuronal excitation through Gq-coupling to TRPC 5 channel activation [16]. Glutamatergic excitation of POMC neurons plays a critical role in regulating energy homeostasis, and the Gq-coupled mGlur1/5 are a critical part of the signaling pathway [7]. *Stim1* mRNA is highly expressed in POMC neurons and in the presence of a store operated Ca2+ channel inhibitor GSK7975A (10 μM) (F). 0.1% v/v DMSO vehicle control had no effect (data not shown). DHPG-induced inward currents were recorded in arcuate YFP labeled neurons (Vhold = −30 mV) from *Pomc*+/− mice. A, the I–V relationship before and during the peak response from the same cell in F indicated that the reversal potential of the nonselective cation current was ~ −30 mV. H, summary of the effects of GSK7975A, HC070 and Pic145 on DHPG-induced current (one-way ANOVA, effect of treatment, F(3, 32) = 16.97, p < 0.0001; Bonferroni’s multiple comparisons test a,b,c, p < 0.05; d, ns.). Scatter plots represent mean ± SEM. n = 11 control, n = 6 HC070, n = 5 pic145 and n = 14 GSK7975A treated neurons.
Figure 5C. After 5 weeks on high fat diet, average 24-hour food intake of Stim1Mut/C6 (32.9 g, n = 8) was significantly more versus the Stim1 in mice on a control diet (10.0 g, n = 11, versus Stim1Mut: 12.9 ± 1.0 pA, n = 10) (Figure 4C).

3.5. Stim1 mutagenesis in PomcCre neurons protects male mice against diet-induced obesity

To assess the impact of Stim1 mutagenesis in ARH-PomcCre neurons on diet-induced obesity, two cohorts of male mice, Stim1Mut (n = 8) and the littermate control PomcCre (n = 8) mice, were put on a HFD for six weeks after one week of the virus injection at around two and half months of age (see Materials and Methods). Over this period, there was significantly less gain in body weight by week 5 in the Stim1Mut versus the PomcCre males (30.4 ± 0.8 g, n = 8 vs. 32.9 ± 0.5 g, n = 8; Figure 5A). Moreover, the average fat mass of Stim1Mut mice was significantly lighter than that of PomcCre controls by week 4 (Stim1Mut vs PomcCre mice fat mass: 5.1 ± 0.6 g, n = 8 vs 7.1 ± 0.8 g, n = 8; Figure 5B). But the lean mass of Stim1Mut mice was significantly more versus the PomcCre mice (Stim1Mut vs the PomcCre mice lean mass: 23.2 ± 0.7 g, n = 8 vs. 22.9 ± 1.7 g, n = 8; Figure 5C). After 5 weeks on high fat diet, average 24-hour food intake was measured in the Stim1Mut group, and the food intake was reduced (Figure 5D). Then both Stim1Mut and PomcCre controls were assessed for glucose tolerance using an intraperitoneal GTT (see Materials and Methods). Both Stim1Mut and PomcCre males started at relatively the same blood glucose levels after an overnight fast (Figure 5E, time 0); and after intraperitoneal glucose injection Stim1Mut male mice did not have significantly different glucose levels compared with PomcCre males, indicating that Stim1Mut males had the same glucose tolerance compared with PomcCre controls. Finally, when both groups were euthanized after 7–8 weeks on HFD and peripheral tissues harvested, the perigonadal fat pad masses were significantly lighter in the Stim1Mut versus the PomcCre males (Stim1Mut vs the PomcCre Pad: 1368 ± 104 mg, n = 8, vs 1935 ± 208 mg, n = 8, Figure 5F). Overall, these results suggest that mutagenesis of Stim1 in POMC neurons affords some protection against diet-induced obesity.

4. DISCUSSION

For the first time, we were able to assess the function of Stim1 in ARH-POMC neurons in the adult nervous system. Quantitative scRT-PCR revealed that we effectively reduced Stim1 mRNA expression by fifty percent, consistent with nonsense-mediated decay following CRISPR/SaCas9 mutagenesis. Reduced Stim1 mRNA in POMC neurons was associated with reduced food intake, attenuated weight gain and reduced body fat and fat pad mass in male mice fed a high fat diet. At the cellular level, Stim1 mutagenesis augmented the depolarizing effects of the mGluR1/5 agonist DHPG, and the TRPC 5 channel blockers HC070 and Pico145 antagonized these excitatory effects of the Gq-coupled receptor. Therefore, utilizing CRISPR technology, we have uncovered a critical role for STIM1 in modulating TRPC 5 channel activity in POMC neurons that ultimately affects the control of energy.
**Figure 5:** *Stim1* mutagenesis in POMC neurons attenuates body mass, fat, and food intake in mice on a high fat diet. Male PomcCre mice were placed on high fat diet (45%) 10 days after being injected with AAV1–FLEX-SaCas9-U6-sgStim1 (n = 8) or AAV1–FLEX-SaCas9-U6-sgControl (n = 8). A, percent change in body weight measured once a week for seven weeks. The high fat diet caused significant weight gain in both groups relative to their baseline, with the controls gaining significantly more by 5 weeks (two-way ANOVA: main effect of treatment (F1, 13) = 7.076, p = 0.0196), main effect of time (F7, 91) = 78.14, p < 0.0001) and interaction (F7, 91) = 5.341, p < 0.0001); control, n = 8, Stim1Mut, n = 8; post hoc Bonferroni test, *p < 0.05, **p < 0.01). Total body fat (B) and Lean mass (C) measured by an EchoMRI Whole Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA). Lean mass does not include bone and fluids within organs. The difference in body fat percentage (B) and lean mass percentage (C) between the groups became significantly different by 4 weeks on high fat diet (two-way ANOVA for B: main effect of treatment (F1, 14) = 3.092, p = 0.0015), main effect of time (F1, 14) = 260.3, p < 0.0001 and interaction (F1, 14) = 3.739, p = 0.0167); control, n = 8, Stim1Mut, n = 8; post hoc Bonferroni test, *p < 0.05. Two-way ANOVA for C: main effect of treatment (F1, 14) = 5.801, p = 0.0304, main effect of time (F1, 14) = 205.1, p < 0.0001 and interaction (F1, 14) = 4.833, p = 0.0452); control, n = 8, Stim1Mut, n = 8; post hoc Bonferroni test, *p < 0.01). D, five weeks after viral injection, average 24-hour food intake was measured for four consecutive days (representative data shown). Stim1Mut significantly reduced food intake (unpaired two-tailed t-test of each animal’s average, t(8) = 2.380, *p = 0.0321). E, six weeks after viral injection and 5 weeks on high fat diet, the GTTs showed no significant difference between the two groups (two-way ANOVA: main effect of treatment (F1, 14) = 0.9184, p = 0.35), main effect of time (F1, 14) = 175.5, p < 0.0001) and interaction (F1, 14) = 0.7049, p = 0.4919); control, n = 8, Stim1Mut, n = 8; post hoc Bonferroni test, *p < 0.05. F, Fat pad of Stim1Mut mice on high fat diet for 7–8 weeks were 30% lighter than control DIO mice at the time that they were euthanized for electrophysiology studies (unpaired two-tailed t-test, t(8) = 2.724, **p = 0.0165).

homeostasis. Furthermore, CRISPR technology enabled Stim1 mutagenesis in an adult animal which not only reduced the chance of developmental compensation, but also avoided apparent embryonic lethality.

Over ten years ago, we discovered that leptin and insulin excite/depolarize POMC neurons through activation of TRPC 5 channels [11,12,44,45]. Leptin binds to its receptor (LRb) to activate Jak2, which phosphorylates insulin receptor substrate (IRS) proteins that activate a PI3 kinase signaling pathway [46]. In POMC neurons the insulin receptor (Insr) couples to PI3K activity [47,48], and the insulin receptor-mediated excitation of POMC neurons is abrogated by inhibition of PI3K activity [11,12,48,49]. Activation of PI3K generates PIP3, which stimulates phospholipase C (PLC) and protein kinase B (Akt) [12,50–52]. Subsequently, PLC hydrolyzes PIP3, which ultimately modulates TRPC 5 channel activity [12,53,54]. In addition, PI3K quickly increases the vesicular trafficking of TRPC 5 channels to the plasma membrane to further boost Ca2+ entry into neurons [55]. Therefore, insulin appears to have dual complementary actions to directly activate TRPC 5 channels and to mobilize intracellular vesicular pools of TRPC 5 channels to further augment its activity via PI3K signaling pathways in POMC neurons [12,56].

More recently, we uncovered a critical role of STIM1 in the insulin signaling cascade in POMC neurons [15]. TRPC channels form either receptor-operated cation channels (activated by membrane delimited receptors) or store-operated calcium channels (activated by depletion of calcium stores), which is dependent on their association with STIM1 and plasma membrane calcium channels (e.g., TRPC and ORAI channels) [8,13,57]. Upon depletion of intracellular (endoplasmic reticulum) Ca2+ stores, STIM1 undergoes a conformational change, oligomerizes and then interacts with plasma membrane ORAI and TRPC channels to become plasma membrane calcium release-activated calcium channels [13,14,58]. Stim1 mRNA is highly expressed in POMC neurons (Figure 1) [15], and estradiol downregulates Stim1 mRNA expression, which is critical for maintaining insulin excitability in POMC neurons with diet-induced obesity [15]. Indeed, in ovarioiectomized females that are relatively refractory to insulin excitation, bath perfusion of a SOCE inhibitor (GSK7975A) rapidly increases the insulin-mediated excitation of POMC neurons (i.e., activation of the TRPC 5 mediated inward current), which supports the concept that TRPC 5 channels play a role both in SOCE and receptor operated calcium entry [8,13]. Therefore, selective mutagenesis of Stim1 in POMC neurons would not only enhance the responses to insulin and leptin by rendering TRPC 5 channels as receptor-operated channels, but also would boost the excitatory responses to glutamate via mGlur1/5 (Figure 6) and serotonin via the 5HT2C receptor [59].

Downregulating Stim1 decreases the SERCA (sarcoplasmic/endoplasmic reticulum Ca2+-ATPase)-dependent cytosolic Ca2+ clearance and elevates intracellular Ca2+ levels [31], which could also contribute to...
Stim1 and in heterologous cells expressing in distribution of TRPC 5 channels across the plasma membrane and resistance with Purkinje neurons [31], we measured a significant decrease in the input resistance with "Stim1" deletion, which we believe reflects an increase in distribution of TRPC 5 channels across the plasma membrane and coupling to mGluR1/5 [8]. A similar scenario occurs in cortical neurons and in heterologous cells expressing Cav1.2 (L-type) calcium channels and "Stim1", where inhibition of Stim1 augments Ca²⁺ influx through L-type calcium channels [62,63]. Furthermore, deletion of "Stim1" in cardiomyocyte-derived (HL-1) cells increases the peak amplitude and current density of T-type calcium channels and shifts the activation curve toward more negative membrane potentials [64]. Biotinylation assays have revealed that deletion of "Stim1" increases T-type calcium channel surface expression, and co-immunoprecipitation assays suggest that Stim1 directly regulates T-type channel activity [64]. Thus, Stim1 appears to dampen the activity of voltage-gated calcium channels. Importantly, estradiol treatment downregulates Stim1 expression in the ARH of ovariectomized female mice and guinea pigs [15], which is why we focused on males in this study. In contrast, E2 upregulates Cav3.1 channel expression and whole cell currents in POMC neurons [65]. Calcium influx via Cav3.1 channels may also facilitate TRPC 5 channel opening in POMC neurons, but this remains to be determined. Besides the peptides kisspeptin, neurokinin B and dynorphin, Kiss1ARH neurons also co-express the vesicular glutamate transporter 2 (Vglut2) [27,66], and we have documented that optogenetic stimulation of Kiss1ARH neurons expressing channelrhodopsin releases glutamate, which is dependent on the estradiotic state of females [7]. Although the mRNA expression of Kiss1, Tac2 and Pdyn mRNA in Kiss1ARH neurons are all down-regulated by E2 [67,68], Vglut2 mRNA expression is upregulated together with increased probability of glutamate release in E2 treated, ovariectomized females [7]. Low frequency (1–2 Hz) optogenetic stimulation of Kiss1ARH neurons evokes fast ionotropic glutamatergic EPSCs in POMC and AgRP neurons, but high frequency (20 Hz) optogenetic stimulation releases enough glutamate to induce a slow excitatory response in POMC neurons but a slow inhibitory response in AgRP neurons [7,27,56]. Indeed, the group I mGluR agonist DHPG depolarizes POMC neurons, while group II/III mGluR agonists hyperpolarize AgRP neurons [7]. As opposed to Group I mGluRs (mGluR1 and mGluR5) coupling to Gαq/11, group II/III mGluRs (mGluR2 and mGlu7) are Gzαi0-coupled [69]. Hence, the output of Kiss1ARH neurons excites the anorexigenic POMC neurons and inhibits the orexigenic AgRP neurons. Therefore, Kiss1ARH neurons appear to be an integral part of an anorexigenic circuit in the hypothalamus [7,70,71] [72], which funnels through TRPC 5 signaling in POMC neurons. Besides the glutamatergic projections from the local ARH (kisspeptin) neurons there are glutamatergic inputs from the ventromedial nucleus that are highly regulated by energy states [73–77] that may also signal via mGluR1/5 and be similarly augmented by Stim1 downregulation. Presently, we have been able to assess a specific function of "Stim1" in ARH-POMC neurons in the adult male. Our attempts to conditionally knockout Stim1 in POMC cells failed perhaps due to the fact POMC is not only expressed in neurons but pituitary corticotrophs [78]. Therefore, we took advantage of CRISPR technology [17,18] utilizing a sgRNA to selectively mutate "Stim1" in adult ARH-POMC neurons in male mice. Importantly, there is not a pharmacological approach to inhibit Stim1 signaling in adult animals (e.g., using the SOCE inhibitor GSK7975A) since Stim1 is ubiquitously expressed both in neurons and non-neuronal cells [13,14]. Reduced Stim1 mRNA expression in POMC neurons was associated with reduced food intake, attenuated weight gain and reduced body fat and fat pad mass in male mice fed a high fat diet. Although we focused on the glutamatergic signaling via mGluR1/5 at the cellular level, the metabolic phenotype would compel one to investigate the effects of insulin and leptin, both of which signal via TRPC5 channels at the plasma membrane [11,12,59]. Indeed, the neuroprotective effects of Stim1 mutagenesis could be driven, in part, by enhanced insulin/leptin signaling in POMC neurons, which needs to be addressed in future experiments. **AUTHOR CONTRIBUTIONS**

JQ performed and analyzed the electrophysiology experiments. MAB did the single cell harvesting and quantitative PCR analysis of the
harvested cells. TLS did the calcium imaging experiments. LSZ and ACH designed and characterized the sgRNA for Stmn1. JO, OKR and MJK designed the experiments, analyzed the data and wrote the manuscript with input from the other authors.

DECLARATIONS OF INTEREST

None.

DATA AVAILABILITY

Data will be made available on request.

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CONFLICT OF INTEREST

None declared.

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