Olfactory bulb-targeted quantum dot (QD) bioconjugate and Kv1.3 blocking peptide improve metabolic health in obese male mice

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Abstract

The olfactory system is a driver of feeding behavior, whereby olfactory acuity is modulated by the metabolic state of the individual. The excitability of the major output neurons of the olfactory bulb (OB) can be modulated through targeting a voltage-dependent potassium channel, Kv1.3, which responds to changes in metabolic factors such as insulin, glucose, and glucagon-like peptide-1. Because gene-targeted deletion or inhibition of Kv1.3 in the periphery has been found to increase energy metabolism and decrease body weight, we hypothesized that inhibition of Kv1.3 selectively in the OB could enhance excitability of the output neurons to evoke changes in energy homeostasis. We thereby employed metal-histidine coordination to self-assemble the Kv1.3 inhibitor margatoxin (MgTx) to fluorescent quantum dots (QDMgTx) as a means to label cells in vivo and test changes in neuronal excitability and metabolism when delivered to the OB. Using patch-clamp electrophysiology to measure Kv1.3 properties in heterologously expressed cells and native mitral cells in OB slices, we found that QDMgTx had a fast rate of inhibition, but with a reduced IC50 and increased action potential firing frequency. QDMgTx was capable of labeling cloned Kv1.3 channels but was not visible when delivered to native Kv1.3 in the OB. Diet-induced obese mice were observed to reduce body weight and clear glucose more quickly following osmotic mini-pump delivery of QDMgTx/MgTx to the OB, and following MgTx delivery, they increased the use of fats as fuels (reduced respiratory exchange ratio). These results suggest that enhanced excitability of bulbar output neurons can drive metabolic responses.
The olfactory bulb (OB) processes olfactory information relayed peripherally from the main olfactory epithelium to communicate to the brain concerning the external chemical environment cued by odorant molecules (Kleene, 2008; Antunes et al., 2014). The integrity of that information can be altered depending upon the nutritional state of the animal or individual, whereby fasting or excess nutrition can modify electrical signaling or the olfactory sensory structures in the OB (Aime et al., 2014; Fadool & Kolling, 2020; Julliard et al., 2017; Palouzier-Paulignan et al., 2012; Thiebaud et al., 2014). One of the drivers of excitability in the major projection neurons of the OB, the mitral cells, is a voltage-gated potassium channel, Kv1.3. Action potential firing frequency, shape, and spike train periodicity are known to be driven by metabolic hormones and important energy factors that decrease Kv1.3 current amplitude, suggesting that the ion channel can differentially regulate excitability based on nutritional status and energy homeostasis (Fadool & Kolling, 2020; Fadool et al., 2000, 2011; Julliard et al., 2017; Kovach et al., 2016; Marks & Fadool, 2007; Marks et al., 2009; Palouzier-Paulignan et al., 2012; Thiebaud et al., 2016, 2019; Tucker et al., 2010a; Tucker et al., 2010b; Tucker et al., 2013). Global gene-targeted deletion of Kv1.3 channel or pharmacological block of the channel peripherally in mice decreases body weight and reduces diet-induced obesity (DIO) (Fadool et al., 2004; Tucker et al., 2008, 2012a, 2012b; Upadhyay et al., 2013; Xu et al., 2003). Hence, our goal was to design a chemical probe to block Kv1.3 specifically in the OB, to determine if mice made obese through diet could lose body weight by changing excitability centrally.

We selected the pore-blocker margatoxin (MgTx) to inhibit Kv1.3 current flow and label cells expressing the channel because of its reported selectivity (Garcia et al., 1997; Garcia-Calvo et al., 1993; Knaus et al., 1995) and applications in the OB (Colley et al., 2004; Fadool & Levitan, 1998; Mast & Fadool, 2012; Schwartz et al., 2017). Luminescent quantum dots (QDs) were utilized because of their intense photo-properties and ability to tune the interactions with biomolecules via modification of the QDs polymer ligand (Sundberg & Bruce Martin, 1974; Wang et al., 2016). QDs have unique optical and spectroscopic properties (Mattoussi et al., 2012; Medintz et al., 2005) that make them advantageous drug vectors for bioimaging. MgTx can be covalently conjugated to polyethylene glycol-modified Zn2+ QDs and still retain ability to inhibit Kv1.3 (Schwartz et al., 2017), so that the conjugated molecule can be visually tracked in vivo. However, controlling the valence and orientation of MgTx on the conjugates is difficult via this strategy (Mout et al., 2012), and significant loss of material during the conjugation limits in vivo applications (Schwartz et al., 2017). We have now, alternatively, produced recombinant MgTx with an N-terminal polyhistidine tag that allows self-assembly of a controlled number of peptides per QD. This approach has previously been reported to allow conjugation of a variety of biomolecules to both QDs and gold nanoparticles and can be attributed to the strong affinity of histidine for metal-rich surfaces (Aldeek et al., 2013a; Aldeek et al., 2013b; Anikeeva et al., 2006; Goldman et al., 2005a; Goldman et al., 2005; Medintz et al., 2006; Medintz et al., 2006; Sapsford et al., 2007; Sundberg & Martin, 1974; Wang et al., 2015a; Wang et al., 2016b). Compared to covalent conjugation, self-assembly driven by metal-His coordination is highly efficient and prevents significant loss of material while providing control over the number and orientation of biomolecules bound to the QD surface (Anikeeva et al., 2006; Goldman et al., 2005a; Goldman et al., 2005b; Medintz et al., 2006a; Medintz et al., 2003, 2004; Medintz et al., 2006b; Wang et al., 2007, 2016a).

Following production of MgTx with an N-terminal polyhistidine tag (HisMgTx) and conjugation of this peptide to QDs using polyhistidine self-assembly (QDMgTx), we electrophysiologically measured the bioconjugate’s ability to inhibit Kv1.3 in a heterologous expression system. We then tested its ability to increase excitability by blocking the native channel in mitral cell neurons of the OB. Owing to our ability to synthesize the bioconjugate in sufficient quantity for in vivo applications, we delivered HisMgTx and QDMgTx to DIO mice using osmotic mini-pumps cannulated into the OB such that we could measure the ability of the inhibitor to modulate whole-body metabolism and confirm targeted inhibition of Kv1.3 in the OB. Block of Kv1.3 in the OB in the obese mice caused a reduction in body weight, modified fuel utilization, and altered ingestive behaviors. These results provide evidence that modulating OB physiology, centrally, through targeting Kv1.3 in the brain can change energy utilization and eating behaviors.

2 | MATERIALS AND METHODS

Our study design was not pre-registered as a clinical trial.

2.1 | Ethical approval

All animal experiments were approved by the Florida State University (FSU) Institutional Animal Care and Use Committee (IACUC) under
protocol #1427 and were conducted in accordance with the American Veterinary Medicine Association (AVMA) and the National Institutes of Health (NIH) guidelines. For preparation of OB slices, mice were anesthetized with isoflurane (Aerrane; Baxter) using the IACUC-approved drop method and were then killed by decapitation (AVMA Guidelines on Euthanasia, June 2007).

### 2.2 | Animal care

All mice (C57BL/6J background strain, Research Resource Identifier, RRID:IMSR_JAX:000644; The Jackson Laboratory) were housed individually in conventional style open cages at the Florida State University vivarium on a standard 12/12 hr light/dark cycle and were allowed ad libitum access to 5,001 Purina Chow (Purina) or a moderately high fat diet (MHF, Cat. #D12266B, Research Diets. 32% fat, 17% protein, and 51% carbohydrate) and water. Because of need for metabolic assessment and surgical recovery, all mice were individually housed and provided with two sources of enrichment—a house and a nestlet. Post-natal day (P) 15–30 mice of both sexes were used for electrophysiology. Mice that were used for metabolic assessment were maintained on MHF diet upon weaning (P24) for an average of 7.5 to 8 months that was not significantly different between treatment groups (Control = 240 ± 20 days, MgTx = 232 ± 19 days, and QDMgTx = 232 ± 21 days; one-way ANOVA, F(2,19) = 0.01547, p = .9847). Assignment of animals to a treatment group was arbitrary and the mean body weight for a treatment group following induction of obesity and prior to surgery was not significantly different (Control = 37.0 ± 1.6 g, MgTx = 35.1 ± 1.5 g, and QDMgTx = 39.2 ± 1.7 g; one-way ANOVA, F(2,19) = 1.411, p = .2682). Because experiments were designed to test the metabolic effect of QD conjugates following diet-induced obesity, only male mice were used in these studies given our previous report that female mice do not gain adiposity or body weight (Tortoriello et al., 2004, 2007). A total of 55 mice were used in our study. Our experiments were exploratory and the study design did not contain pre-determined exclusion criteria. Four mice were excluded in the study’s results (one was excluded as a statistical outlier (two-sided Grubbs’ test), two developed dermatitis as a result of oily fat diet, and one did not survive surgery) and an additional five mice were used to optimize placement of the osmotic minipump using stereotactically guided surgery. The number of subjects per experiment is specified in the figures and figure legends. Animal subjects were not randomized, rather assignment of animals to a treatment group was arbitrary—the surgeon blindly selected which pump to implant while a separate investigator loaded and retained the knowledge of pump contents. Each of the pumps (phosphate-buffered saline [PBS] control, HisMgTx, and QDMgTx) had an equal chance of being selected. Animals were metabolically screened in cohort sizes of four because this number of surgeries was achievable in a day’s duration. Each day contained mouse surgery using all treatment groups so that data collection was not biased.

### 2.3 | Solutions and reagents

All salts and sugars were purchased from Sigma-Aldrich or ThermoFisher Scientific. Solutions used for expression and purification of the His-conjugated margatoxin peptide (HisMgTx) included Luria broth (LB, Cat. #244620; BD Biosciences) supplemented with 1% glucose, 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG, #5800; EM Science), equilibration buffer (25 mM Tris, 300 mM NaCl), 50 mM EDTA, 100 mM NiSO₄, and 300 mM imidazole. PBS was used for conjugation (pH 8.0), prepared as previously described (Biju et al., 2008). For whole-cell electrophysiology experiments using human embryonic kidney 293 cells (HEK293 cells), the bath solution consisted of (in mM): 150 NaCl, 5 KCl, 2.6 CaCl₂, 2 MgCl₂, and 10 Hepes, pH 7.4; 305 millimol (mOsm). The intracellular pipette solution consisted of (in mM): 145 KCl, 10 HEPES, 10 EGTA, 2 MgCl₂, 3 NaATP, 0.4 GTP, pH7.4; 295 mOsm. The artificial cerebrospinal fluid (ACSF), sucrose-modified ACSF, and intracellular pipette solution for slice electrophysiology experiments were carefully monitored for osmolarity and prepared as previously reported (Fadool et al., 2011; Tucker et al. 2013; Thiebaud et al., 2016).

### 2.4 | cDNA constructs

All Shaker channel coding regions were downstream from a cytomegalovirus promoter. Rat Kv1.3 (rKv1.3) was subcloned into the multiple cloning region of pcDNA3 (now available only as 3.1; RRID:Addgene_11588) and subcloned into the pcDNA 3 vector between the BamHI and EcoRI restriction sites as previously described (Holmes et al., 1996). DNA encoding human CD8 was amplified from pCDM8 (RRID:Addgene_11588) and subcloned into the pcDNA3 vector between the BamH1 and EcoR1 restriction sites (Mast et al., 2010). DNA encoding MgTx was subcloned into a PET-28b vector (Cat. # 69865-3; EMD Biosciences) containing a His₆ tag on the N-terminus.

### 2.5 | Experiment 1. Quantum dot (QD) synthesis and conjugation to margatoxin (MgTx)

#### 2.5.1 | QD growth and phase transfer

The luminescent CdSe-ZnS core-shell quantum dots (QDs) used in this study had an approximate diameter of 6 nm and emitted in the green region of the visible spectrum (peak at 533 nm) (Clapp et al., 2006; Yu & Peng, 2002). These nanocrystals were grown stepwise, starting with the core, followed by ZnS overcoating, using high-temperature reduction of cadmium, selenium, zinc, and sulfur precursors in coordinating solvent mixtures made of alkylphosphines, alkyphosphate-carboxyl, and alkylamines, following previous protocols (Clapp et al., 2006). The native capping ligands, made of a mixture that included trioctylphosphine/thrioclyphosphineoxide (TOP/TOPO) and alkylphosphonic acid, were exchanged with poly(isobutylene-alt-maleic anhydride)
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(PIMA)-based polymer ligand presenting several imidazole and zwitterionic moieties (His-PIMA-ZW) to promote dispersion of the QDs in buffer media and allow conjugation to His-biomolecules. Additional details on the phase transfer, conjugation steps, and purification of the conjugates can be found in previous reports (Wang et al., 2016; Wang et al., 2015). The final concentration of the QD dispersion was determined from the absorbance at 350 nm, using an extinction coefficient of $2.467 \times 10^{-6}$ M$^{-1}$cm$^{-1}$ for the QDs (Leatherdale et al., 2002).

2.5.2 | Recombinant expression of HisMgTx

His-conjugated margatoxin (HisMgTx) was subcloned into the multiple cloning region of the pET-28b+ vector (EMD Biosciences, Cat. #69865-3) using Ndel and Xhol restriction sites (Addgene Vector Database). The construct was transformed into BL21 Shuflle T7 Express Escherichia coli (New England BioLabs, Cat.#C3029J). The pET-28b vector included an N-terminal His$_6$ tag, which allowed for affinity purification and conjugation to QDs and a thrombin cleavage site (underlined) between the His$_6$ tag and the MgTx coding sequence (MGSSHHHHHH-SGGGTPRGSS-HMTIINVKCTSPKQCLP PCKAQ FGQSAGAKCMNGKCKCYPH). BL21 Shuflle T7 Express E. coli contain an oxidizing cytoplasm that promotes proper disulfide bond formation in HisMgTx. Transformed E. coli were grown in LB with 1% glucose at 37°C to OD$_{600}$ = 0.8 while shaking at 250 RPM, then expression was induced with 0.5 mM IPTG at 16°C while shaking at 250 RPM (Figure 1a). Following overnight expression, bacterial cells were lysed using a microfluidizer (110L; Microfluidics), then HisMgTx was purified from the clarified lysate using nickel affinity chromatography (Gold Biotechnology, #H-320-50) followed by reversed-phase high-performance liquid chromatography (RP-HPLC), as shown in Figure 1b. RP-HPLC (Shimadzu Prominence HPLC) was performed with a C$_{18}$ analytical column (Cat. #651286-8; Phenomenex) using a flow rate of 1 ml/min and a gradient of 15%–30% solvent B (0.075% trifluoroacetic acid (TFA) in 90% acetonitrile) in solvent A (0.1% TFA in water) over 50 min. HisMgTx was detected using the peptide absorbance at 220 nm. The monoisotopic mass of the purified recombinant HisMgTx determined using mass spectrometry was 6,475.9 kDa (Figure 1c). The purified HisMgTx was quantified using a Qubit Fluorimeter (Model #Q32857; ThermoFisher Scientific), then lyophilized (Model #MODULYOD-115; ThermoFisher Scientific) and stored at −20°C. Prior to use, the peptide was reconstituted in PBS with 0.05% bovine serum albumin (BSA).

2.5.3 | Preparation of QDMgTx

HisMgTx was conjugated to QDs (referred to as QDMgTx) by mixing the peptide with His-PIMA-ZW capped QDs in PBS followed by incubation at 4°C for 1 hr. Addition of the His$_6$ tag to

FIGURE 1 Purification of HisMgTx from T7 Shuffle Express E. coli. (a) 20% acrylamide gel illustrating various stages in the expression and purification of recombinant HisMgTx. Pre = pre-induction sample, Post = post-induction sample, L = cell lysate, S = supernatant, P = pellet, W1 = wash 1 from affinity column, W2 = wash 2 from affinity column, E = elution with 50 mM imidazole. Arrow = eluted protein product. (b) Reversed-phase high-performance liquid chromatography (RP-HPLC) chromatogram with single peak at ~23 min retention time corresponding to recombinant HisMgTx. (c) LC-MS/MS spectrum of purified HisMgTx, showing a monoisotopic mass of 6,475.85 Da. HisMgTx, histidine epitope-tagged margatoxin
the N-terminus of MgTx allowed facile binding to Zn$^{2+}$ as a result of high affinity of histidine for Zn$^{2+}$. Altered electrophoretic mobility of QDMgTx in comparison to non-conjugated QDs on a 0.8% agarose gel confirmed successful interaction of HisMgTx with the QDs (Figure 2). For the agarose gel experiments, several QDMgTx dispersions were prepared where the molar ratio of QD:HisMgTx varied from 1:10 to 1:60. QD concentration was maintained at 100 nM. For all cell labeling, electrophysiology, and animal studies, the QDMgTx was prepared at molar ratio of 1:15 and concentrations described for QDMgTx are with respect to the QD.

2.6 | Experiment 2. Using heterologously expressed Kv1.3 channels to test QD targeting and function

2.6.1 | Maintenance and transfection of HEK293 cells

We used Human Embryonic Kidney Cells (HEK293; RRID:CVCL_0045) that are not listed as a commonly misidentified cell line at the International Cell Line Authentication Committee Registry (ICLACR). Our HEK293 cells were contributed by Dr. Robert Margolski (Monell Chemical Senses Center) and have been authenticated for cross-contamination and misidentification by profiling short tandem repeats. Moreover, cell culture practices at Florida State University have followed “Guidance on Good Cell Culture Practices (GCCP)” (Coecke et al., 2005). Our last cryopreserved batch of HEK293 cells was prepared in February of 2016 and each vial is experimentally used between passages 4 and 25. HEK293 cells were grown in Modified Eagle’s Medium (MEM, Cat. #12360-038; ThermoFisher Scientific/Gibco) supplemented with 2% penicillin/streptomycin (Cat. #P0781; Sigma) and 10% fetal bovine serum (Cat. #16000-044; ThermoFisher Scientific/ Gibco). Plating to low density and transfection procedures were as previously described (Cook & Fadool, 2002). Briefly, when cells reached 70%–80% confluency they were transiently cotransfected with 0.3 μg Kv1.3 and 0.2 μg pCDM8 cDNAs for 4 hr using lipofectamine (Cat. #18324-012; ThermoFisher Scientific/Invitrogen) in serum-reduced media (OptiMEM, Cat. #31985-070; ThermoFisher Scientific/Gibco). Including cDNA encoding the human CD8 transmembrane protein permitted panning for transfected cells by labeling cells with a red polypropylene antibody-linked bead prior to patch clamping (Mast & Fadool, 2012). The investigator was not blind to which cDNA was transfected into the HEK 293 cells.

FIGURE 2 Conjugation of HisMgTx to green CdSe-ZnS core-shell quantum dots (QDs). These QDs were modified with a polyimidazole-based zwitterionic ligand containing the short-chain poly(isobutylene-alt-maleic anhydride) (His-PIMA-ZW). (a) Schematic representation of a QD modified with His-PIMA-ZW (Wang et al., 2016) and of the same QD with HisMgTx bound to its surface. (b) 0.8% agarose gel confirming HisMgTx binding to QDs (20 min run time). As a larger ratio of HisMgTx is bound to the surface, the constructs’ mass increases. [QD] =100 nM. (+) and (−) indicate positive and negative poles of gel. (c) Same as (b), however, the sample was electrophoresed for longer (70 min) and includes HisMgTx (MgTx = HisMgTx) and bovine serum albumin (BSA) (carrier protein for HisMgTx). (d) Coomassie stain of (c), to probe the presence of peptide not bound to QDs following conjugation (MgTx = HisMgTx)
2.6.2 | Electrophysiology of HEK293 cells

Thirty-six hours post-transfection, cells were incubated with Dynabeads® CD8 microparticles (Cat. #11147D; Thermo Fisher Scientific/Gibco) for 2 min to visualize channel-transfected cells (Mast & Fadool, 2012). Hoffman modulation contrast optics was used to visualize cells at 40× magnification (Axiovert 135; Carl Zeiss). Patch electrodes were fabricated from Sutter glass (BF150-86-15; Sutter Instrument Co.) using a vertical puller (Model PP-830; Narishige) to achieve pipette resistances of 7–10 MΩ. Outward currents were recorded at room temperature (RT; 20–22°C) as acquired from the whole-cell configuration using an Axopatch 200B patch-clamp amplifier (Molecular Devices/Axon Instruments). All voltage signals were generated and data acquired with the use of an Axon Digidata 1,200 A/D converter in conjunction with pClamp v10.3 software (Molecular Devices/Axon Instruments). Recordings were filtered at 2 kHz and digitized at 2–5 kHz. Typically, cells were held (Vh) at −90 mV and stepped to a depolarizing potential of +40 mV (Vp) using a pulse duration (Pd) of 250 ms. Cells were stimulated with an interpulse interval of 45 s to prevent cumulative inactivation, a property of Kv1.3 channels (Cook & Fadool, 2002; Kupper et al., 1995; Marom & Levitan, 1994). HisMgTx, QDs, or QDMgTx were applied directly to the bath to measure change in Kv1.3 current properties in response to peptide inhibitor, nanoparticle, or conjugate.

2.6.3 | Nano-labeling of Kv1.3 channel in HEK293 cells

HEK293 cells were grown on 12-mm glass coverslips (Cat. #12-545-90; Thermo-Fisher Scientific) and were transfected with Kv1.3 or pcDNA3 CDNA at 70%–80% confluency. At 48 hr post-transfection, cells were washed twice with HEK293 bath solution to ensure that channels were in an open state so that QDMgTx could bind to the open pore of the channel. Cells were then incubated with the QDMgTx conjugate in PBS (1:15; 50 nM QD, 750 nm HisMgTx) for 30 min, permeabilized with 0.2% Triton X-100 in PBS (Cat. #9002-93-1; Thermo Fisher Scientific) for 10 min, fixed with freshly prepared 4% paraformaldehyde in PBS (Thermo-Fisher Scientific, Cat. #BPS31-25) for 10 min, and then nuclear stained with 4,6-diamidino-2-phenylindole (DAPI, Cat. #D1306; Thermo-Fisher Scientific). All incubations were performed at RT. Cells were rinsed with PBS twice between each step. Coverslips were mounted to glass slides for imaging using Fluoromount-G mounting media (Cat. #1405002) to a pipette resistance of 4–7 MΩ. Following determination of spike threshold, cells were stimulated with a long, perithreshold current step (typically ranging from 5 to 50 pA) for 5,000 ms every 10 s to acquire spike frequency data under ASCF conditions (Control). After recording for a minimum of 10 min, the bath was switched to QDs in ASCF (QD) and spike firing frequency was measured using the same current injection for 10 min. Finally, the bath was switched a third time to apply QDMgTx to the slice and the spike firing frequency was again measured for a minimum of 10 min. The final working concentration of QDMgTx added to the bath for these slice experiments was 10 nM.

2.7 | Experiment 3. QD functional effect on olfactory bulb using ex vivo slice electrophysiology

P21–P35 C57Bl/6J mice were anesthetized by inhalation of isoflurane (see Ethics section) and quickly decapitated, and then the OBs were exposed by removing the dorsal and lateral portions of the skull between the lambda suture and cribriform plate. The OBs were harvested and 300-μm coronal sections prepared using ice-cold oxygenated sucrose-modified ACSF solutions during vibratome sectioning (Vibratome/Leica Model 1000, Wetzlar, Germany), as previously described (Fadool et al., 2011; Thiebaud et al., 2016). Then slices were allowed to recover in an interface chamber containing oxygenated ACSF (Krimer & Goldman-Rakic, 1997) for 20–30 min at 33°C and then were maintained at RT for 60 min before recording. Recordings from OB slices were made at RT using a continuously perfused (Ismatec; 1 ml/min), submerged-slice recording chamber (RC-26; Warner Instruments) with ASCF. Slices were visualized at 10 × and 40 × using an Axioskop 2FS Plus microscope (Carl Zeiss Microimagining, Inc.) equipped with infrared detection capabilities (Dage MT1, CCD100). Electrodes were fabricated from borosilicate glass (Hilgenrein Cat. #1405002) to a pipette resistance of 4–7 MΩ. Following determination of spike threshold, cells were stimulated with a long, perithreshold current step (typically ranging from 5 to 50 pA) for 5,000 ms every 10 s to acquire spike frequency data under ASCF conditions (Control). After recording for a minimum of 10 min, the bath was switched to QDs in ASCF (QD) and spike firing frequency was measured using the same current injection for 10 min. Finally, the bath was switched a third time to apply QDMgTx to the slice and the spike firing frequency was again measured for a minimum of 10 min. The final working concentration of QDMgTx added to the bath for these slice experiments was 10 nM.

2.8 | Experiment 4. QDMgTx/HisMgTx effect on metabolism determined using Comprehensive Laboratory Animal Metabolic System

2.8.1 | Osmotic mini-pump surgery

Mice were weaned at P24 to MHF diet for a mean of 7.5–8 months prior to surgical placement of an osmotic mini-pump to deliver QDMgTx/HisMgTx or control treatments. We assembled cannulated
Alzet micro-osmotic mini-pumps (Cat. #1002; Durect Corporation) to allow for continuous delivery at a rate of 0.25 μl per hour over a 14-day period. The mini-pumps were filled with control PBS (2% BSA), 375 nM HisMgTx, 25 nM QD, or 25 nM QDMgTx 1:15 diluted PBS (2% BSA), and were prepared as per manufacturer’s protocols the evening prior to surgeries. Animals were anesthetized with a continuous flow of isoflurane and were given 0.1 mg/kg buprenorphine HCl (Cat. #NDC42023-179-01; PAR Pharmaceuticals) for pain management. Mice were restrained in a Stoelting/Kopf Instruments stereotoxic apparatus and two holes were drilled (Stoelting, micromotor high-speed drill model 51,449) into the skull of the animal above the OB at the mitral cell layer coordinates (AP −0.25 mm from bregma, M/L ±0.075 and D/V −2.25 mm from dura). Cannula (Cat. #3280PD-1.5-SPC OP CONN28 gauge DBL; Plastics One) were bilaterally inserted (inter-cannula distance = 1.5 mm; cannula depth = 2.25 mm) into the holes and then a fitted connector (21Y connector Cat. # 37,200 81003720001F; Plastics One) delivered the pump contents to the cannula. The mini-pump itself was inserted in a pocket created under the skin on the back of the animal. The cannula were adhered to the skull using cement and the skin was closed on the back and neck using surgical staples (Clay Adams wound clips Cat. # 427,631, 9 mm; Becton Dickinson). Mice were closely monitored on a heating pad in their home cage while recovering from initial anesthesia and were administered a second dose of buprenorphine HCl under anesthesia during the first 12 hr of recovery. Mice were allowed to fully recover for 24 hr in the Comprehensive Laboratory Animal Metabolic System (CLAMS; Columbus Instruments; RRID:SCR_016718) home cages, where they were provided moistened food out of the hopper on the cage floor.

Following CLAMS assessment, mice were histologically prepared to confirm cannula placement. Briefly, mice were anesthetized with a ketamine/xylazine mix (80 mg/kg and 10 mg/kg) to reach anesthetic plane 2, and then killed via intracardial perfusion with PBS and 4% paraformaldehyde (Cat. #411678; ThermoFisher Scientific). The skulls were post-fixed in 4% paraformaldehyde/PBS overnight and decalcified for 6–9 days in 0.3 M EDTA (Cat. #5311; ThermoFisher Scientific). Extracted brains were cryoprotected in 10% then 30% sucrose/PBS at 4°C before being frozen in OCT compound embedding medium (Cat. #4585; Fisher Healthcare). Frozen tissue was stored at −80°C until day of cryosectioning. Cryosectioning was performed on a LEICA model CM1850 cryostat (Leica Microsystems) to yield OB coronal sections at 16 μm thickness that were transferred to 1% gelatin-coated Superfrost slides (Cat. #48311, VWR International) and stored at −20°C. OB sections were stained with cresyl violet as previously described (Fadool et al., 2000), then viewed by light microscopy (Zeiss Axiovert, Model #S100) to confirm cannula precision.

2.8.2 Glucose tolerance, indirect calorimetry, and metabolic assessment

The experimental workflow of the glucose tolerance testing, CLAMS metabolic assessment, and surgical implantation of osmotic mini-pumps is diagramed in Figure 6a. The strategy was to perform an intraperitoneal glucose tolerance test (IPGTT) prior and subsequent to drug delivery and allow mice to acclimate to the CLAMS chambers prior to surgery to achieve a baseline, thereby collecting pre- and post-metabolic parameters in response to the QD drug delivery within subjects. For the IPGTT, mice were fasted during the dark phase for 12 hr and then tail blood samples were acquired to monitor clearance of serum glucose over time in response to 2.5 μg/ml glucose per kg body weight (University of Virginia Vivarium Protocols, Susanna R. Keller). Glucose levels were measured using an Ascensia Contour Blood Glucose Monitoring System (Ascensia Diabetes Care US, Inc.).

Metabolic parameters, oxygen consumption (VO₂; ml/kg/min), carbon dioxide production (VCO₂; ml/kg/min), respiratory exchange ratio (RER), energy expenditure (EE; kcal/h), locomotor activity, and caloric and water intake were measured using the CLAMS system (Bell & Fadool, 2017; Fadool & Kolling, 2020). Mice had ad libitum access to food and water in overhead feeders attached to electronic balances that could detect both disturbance (meal duration) and decrease in food mass (meal size). The design of the feeder (Cat. # CLAMS—HC, Homecage) allows placement of the food in a familiar location, and in pellet form, so that there is a catch tray that accounts for weight of crumbs with minor spillage. VO₂ and VCO₂ were normalized with respect to body weight in kilograms. RER was calculated as VCO₂/VO₂. EE was calculated according to the Lusk Equation (3.815 + 1.232 × RER) × VO₂ (Lusk, 1924). Further information concerning the calculation of these metabolic variables by the CLAMS can be found online as a helpful resource (https://www.slideshare.net/InsideScientific/measuring-energy-balance-in-mice-from-vo2vco2-food-intake-and-activity-data). Locomotor activity was continuously recorded using optical beams along the x-axis of the cage so that consecutive photo-beam breaks could be scored as ambulatory movement. All data were recorded in intervals using Oxymax software (CLAX; Columbus Instruments) and each interval measurement represented the average value during a 30 s sampling period per cage. In total, mice were housed in the CLAMS for 19 days (~4 to + 14; see Figure 6a). No data were acquired in the CLAMS during the first 2 days in the chambers (~4 to ~2) to permit acclimation to the environment or during the 24 hr recovery from surgery (0 to 1). The 48 hr after acclimation was used for baseline determination (~2 to 0) and this average was used to normalize data acquired for individual mice for the 11 days subsequent to drug delivery (1 to 11). Data are graphed in a normalized fashion in order to visually compare the response of drug treatment across mouse subjects for a particular metabolic variable, and non-normalized data were used in a paired-subject analysis (repeated measure) as described in the statistical analyses below.

2.9 Data and statistical analyses

Statistical analyses were performed using Prism 8 software (Graph Pad). This software tests for normality using the D’Agostino-Pearson
normality test (omnibus K2). Significantly different means were calculated at the 95% confidence level or $\alpha \leq 0.05$, unless otherwise specified. Sample sizes of our studies were determined by A Priori Type Power Analysis applied for ANOVA and $t$ test, and tested for a range of estimated means and SD for biophysical and metabolic parameters that were adequate for a power of 0.80, $\alpha \leq 0.05$, G*Power Cohen ranges $f \leq 0.68–0.81$.

2.9.1 | Experiment 2a

In order to quantify the fluorescent intensity of QDMgTx binding to Kv1.3-transfected HEK293 cells, images at 40x magnification were analyzed and averaged across a total of five different transfection rounds. A F-max test for homogeneity of variance was performed and then a Student’s $t$ test was applied to determine if there were significantly different means in the fluorescent intensity of mock-transfected cells (pcDNA3 alone) versus that of Kv1.3-transfected cells.

2.9.2 | Experiment 2b

Electrophysiological records were analyzed using Clampex v10.6 software (Molecular Devices/Axon Instruments), Origin v8.0 (Microcal; Borland International), and Excel v2013 (Microsoft Office). HEK293 cells were allowed to stabilize for 3–5 sweeps (Microcal; Borland International), and Excel v2013 (Microsoft Office). HEK293 cells were allowed to stabilize for 3–5 sweeps following stabiliza-

3 | RESULTS

3.1 | QD synthesis and conjugation to MgTx

3.1.1 | Production of recombinant HisMgTx

MgTx was previously produced via recombinant expression in the bacterium E. coli and the yeast Pichia pastoris (Anangi et al., 2012; Garcia-Calvo et al., 1993). We explored production via P. pastoris and chemical synthesis, and constructs prepared using these methods were used for preliminary experiments. However, recombinant expression in E. coli proved to be the most efficient method for our purposes. Here, recombinant HisMgTx was produced using a pET-28b vector transformed into BL21 SHuffle T7 Express E. coli. The pET-28b vector enabled expression of MgTx with an N-terminal His$_6$ tag, which was used for affinity purification and QD binding. An acrylamide gel summarizing the expression and affinity purification is shown in Figure 1a, and the affinity-purified product was purified to homogeneity using C18 RP-HPLC (Figure 1b). The mass of HisMgTx estimated from the gel is higher than expected (~9 kDa), likely because of its high isoelectric point (pI 9.46) reducing its electrophoretic mobility. The mass of the peptide determined using LC-MS/MS (Figure 1c) was found to be equivalent to the predicted mass (experimental = 6,475.85 Da, predicted = 6,473.53 Da).

3.1.2 | Metal-histidine–mediated self-assembly of QDMgTx

Metal-histidine–mediated self-assembly of QDMgTx allowed control over the orientation as well as the valence of peptide per QD. The addition of a terminal His$_6$ tag is commonly used during protein expression in bacteria or during protein synthesis, thus, can be easily applied to conjugate these biomolecules to QDs via polyimazole-to-metal coordination (Dennis et al., 2010; Zhan et al., 2013). Here, we used QDs capped with a His-PIMA-ZW ligand, a compact capping ligand that allowed HisMgTx to directly access the QD surface (Figure 2a) as previously noted (Wang et al., 2015). The conjugation was characterized using gel electrophoresis in which greater amounts of QD bound to HisMgTx were identified as increased mass (Figure 2b). A decrease in mobility of QDs was observed with an increase in the amount of HisMgTx-conjugated per QD. Negligible migration of conjugates was observed as the molar ratio of QD:HisMgTx was increased to and above 1:30, suggesting that the QD surface was saturated with peptide. In Figure 2c,d, the gel was stained with Coomassie blue to identify any unbound peptide
following the conjugation. A lane containing HisMgTx alone or BSA alone (carrier protein for HisMgTx) served as controls. No HisMgTx bands were observed in the 1:5, 1:10, or 1:15 conjugates, indicating that all peptide used for the conjugation was bound to the QDs. HisMgTx bands were observed in the 1:20 and 1:25 conjugations, indicating that following conjugation in these conditions, there was excess peptide not bound to the surface of the QDs. This is in agreement with previous studies in which metal-histidine–mediated self-assembly was similarly used (Medintz et al., 2004). Because the 1:15 QDMgTx conjugate had the largest amount of bound HisMgTx with no observable unbound peptide, our electrophysiological and metabolic analyses used this version of the conjugate.

3.2 Testing QD targeting and function using heterologously expressed Kv1.3 channels

3.2.1 QDMgTx can label HEK293 cells expressing Kv1.3

Kv1.3- or pcDNA3-transfected HEK293 cells were incubated with bath-applied QDMgTx to determine labeling of the conjugate in Kv1.3-transfected cells. QDMgTx labeling was strongly observed in Kv1.3-transfected cells (Figure 3a) and less so under pcDNA3 transfection conditions (Figure 3c). The conjugate appeared to non-specifically bind to pcDNA3-transfected cells with a significantly lower fluorescence intensity than that of Kv1.3-transfected cells (Figure 3d; 79.7 ± 10.4 for pcDNA3 versus. 129.0 ± 18.0 for Kv1.3, n = 5, Student’s t test, p = .0227). When Kv1.3-transfected cells were incubated with QDs alone (Figure 3b), no measurable labeling was observed.

3.2.2 Inhibition of Kv1.3 by HisMgTx and QDMgTx

MgTx is reported to block Kv1.3 with an IC$_{50}$ of 11.7–119 pM (Garcia-Calvo et al., 1993; Knaus et al., 1995), and therefore we measured the ability of HisMgTx and QDMgTx to block Kv1.3 in HEK293 cells (Figure 4). Addition of the epitope tag lowered the affinity of the peptide inhibitor, but HisMgTx still blocked Kv1.3 with picomolar affinity (IC$_{50}$ = 487 ± 106 pM, Hill coefficient = 1). Compared to HisMgTx, the 1:15 QDMgTx blocked Kv1.3 at a lower, but still picomolar affinity (IC$_{50}$ = 825 ± 260 pM, Hill

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**FIGURE 3** Quantum dot margatoxin conjugate (QDMgTx) can label HEK293 cells expressing Kv1.3. Photomicrographs of HEK293 cells transfected with Kv1.3 and incubated with (a) 50 nM QDMgTx 1:15 or (b) 50 nM QDs. (c) Photomicrographs of HEK293 cells transfected with pcDNA3 and incubated with 50 nM QDMgTx, scale bar = 10 μm. (d) Box plot and (e) enlarged photomicrographs to quantify/visualize fluorescence intensity of the labeling in experiments (a) and (c), respectively. Box determined by the 25th and 75th percentiles and whiskers indicate 5th and 95th percentiles; n = 5 transfection batches for each condition. Student’s t test, *Significantly different, p = .0227
coefficient = 1; Figure 4b,d). When applied at equal concentrations (10 nM), HisMgTx and QDMgTx each significantly decreased Kv1.3 peak current amplitude (Figure 4e; two-way mixed-design analysis of variance (ANOVA) using time as a factor, $F(1,50) = 20.61$, $p < .0001$, and using treatment as a factor, $F(3,50) = 3.26$, $p = .0290$). Application of QD alone or control bath solution did not elicit a decrease in voltage-activated current over time ($I_n/I_i$; Figure 4e). The mean percent inhibition (at $I_n/I_i$) by HisMgTx versus QDMgTx was not significantly different ($85 \pm 3.9\%$ for HisMgTx, $n = 5$ versus $73 \pm 7.4\%$ for QDMgTx, $n = 4$, ArcSin Transformation Student’s t test, $p > .05$; Figure 4e). We measured the time required to achieve 50% inhibition of voltage-activated currents in the whole-cell patch configuration to be less than 2 min for 10 nM HisMgTx or QDHisMgTx, and the times were not significantly different (Figure 4f, 32.7 $\pm$ 10.9 s for HisMgTx, $n = 5$ versus. 73.4 $\pm$ 17.7 s QDMgTx, $n = 4$, Student’s t test, $p > .05$).
3.3 | QD functional effect on OB using ex vivo slice electrophysiology

3.3.1 | QDMgTx enhances mitral cell action potential (AP) firing in olfactory bulb slices

The major output neuron of the OB is the mitral cell, with Kv1.3 making up 60%-80% of outward currents in these projection cells (Fadool & Levitan, 1998). When applied via bath application, QDMgTx significantly increased the AP firing frequency over that of control ACSF or QDs (Figure 5; one-way repeated measure (RM) ANOVA, F(2,15) = 8.88, **p = 0.0029). Spike analysis demonstrated that QDMgTx and QDs decreased the pause duration between AP trains (Control, 2,511.0 ± 477.9 ms, n = 46; QD, 1,270.0 ± 77.9 ms, n = 97; QDMgTx; 1,069.0 ± 93.7 ms, n = 43; one-way RM ANOVA, F(2,183) = 6.304, p = .0167), while QDMgTx but not QD increased the interspike interval (ISI) within the AP train (Control, 57.4 ± 4.7 ms, n = 73; QD, 59.6 ± 8.1 ms, n = 116; QDMgTx; 68.0 ± 12.4 ms, n = 61; one-way RM ANOVA, F(2,247) = 6.304, p = <.0001). Spike analysis was acquired over a subset of 2-4 bursting cells (Control, n = 4; QD, n = 3; QDMgTx, n = 2). Previous reports show that MgTx (Mast & Fadool, 2012; Thiebaud et al., 2016) and other neuromodulators (Fadool & Levitan, 1998) when applied via bath application, have less effect on ISI.

Instead of using the IND technique, we used stereotaxically guided placement of osmotic mini-pumps to assure successful and targeted delivery of QDMgTx/HisMgTx and controls to the OB. The experimental work for the glucose tolerance testing, CLAMS metabolic assessment, and surgical implantation of osmotic mini-pumps is diagramed in Figure 6a as previously described in the methods. Each obese mouse was studied for 22 days (~7 to +14) with surgical placement of the pump after 7 days (0) (Figure 6b). Histological examination of the brains following metabolic testing was performed (Figure 6c) to confirm proper placement of the cannula using cresyl violet staining. One mouse was excluded from the data set as a result of a mistargeted cannula. In a separate cohort of non-obese mice (n = 3) for which cannula were clearly perforating the OB, no fluorescent QD labeling could be observed in the OB or anywhere in the brain, despite the empty osmotic capsule (Figure 6d). This result suggested that the drug treatments were osmotically delivered, but were beyond the resolution of detection of the fluorescent label.

3.4 | Assessment of QDMgTx/HisMgTx effect on metabolism using CLAMS

3.4.1 | Delivery of a Kv1.3 inhibitor to the OB of obese mice leads to a reduction in body weight and enhanced glucose clearance

Body weight and glucose clearance were monitored in obese mice receiving PBS (control), HisMgTx, or QDMgTx via cannulated delivery to the OB. HisMgTx- and QDMgTx-treated mice had a significantly reduced body weight compared to PBS control mice (Figure 7a; paired t test, p < .05). We compared the normalized change in body weight over time (+2 to +12) following implantation of the cannulated osmotic mini-pump (at 0; Figure 7b; two-way RM ANOVA, using time as the factor: F(10,190) = 8.367, ****p < .0001). There was a significant interaction of time x treatment: F(20, 190) = 1.923, *p < .05. An IPGTT was performed twice on each mouse (~6, +13) to monitor their fasted ability to clear glucose prior to (Figure 7c) and after drug delivery (Figure 7d). Prior to drug treatment (~6), all mice cleared glucose similarly (two-way RM ANOVA, p > .05). Following drug treatment (+13), mice receiving HisMgTx or QDMgTx cleared glucose significantly faster than control animals (Figure 7d; two-way RM ANOVA using treatment: F(2, 19) = 5.318, *p = .0146 and time: F(6,114) = 53.47, ****p < .0001 as factors). The integrated area under the curve (AUC) is an additional measure of an animals' ability to clear glucose, and therefore we also examined how iAUC changed following drug delivery. Comparison of the iAUC
before (−6) and following (+13) drug delivery is shown in Figure 7e as a normalized change (IPGTT AUC delta). Mice receiving HisMgTx or QDMgTx had a significant change in iAUC (Figure 7e; two-way RM ANOVA using time as the factor: $F(2,15) = 19.84$, ***$p = .0003$), whereas control animals had no change in iAUC ($p > .05$).

### 3.4.2 Inhibition of Kv1.3 in the OB of obese mice does not alter the EE but reduces RER

Energy expenditure (EE), or the amount of calories burned per hour, is a compilation of resting metabolic rate, the thermic effect of eating, and energy expended during voluntary and involuntary physical movement. Following delivery of HisMgTx or QDMgTx (+2 to +12), there was no change in EE in either the light or dark cycle (Figure 8a,b; two-way RM ANOVA using treatment as the factor: light cycle: $F(2,15) = 0.7349$, $p = .496$; dark cycle, $F(2, 15) = 1.484$, $p = .2581$). It is well known that EE can be transiently increased because of the trauma or inflammation resulting from surgery (Clark, 1973) and we observed this increase in the light cycle following surgery on Day 0 (not shown; 0.20- to 0.28-fold increase) and a subsequent lowering of EE over time ($F(9, 135) = 2.60$, *$p = .0085$) for all treatments without a significance in the Bonferroni's post hoc test ($p > .05$). VO$_2$ is also a primary measure of metabolism. We observed no difference in VO$_2$ with respect to delivery of HisMgTx or QDMgTx (+2 to +12), in either the light or dark cycle (Figure 8c,d; two-way RM ANOVA using treatment as the factor: light cycle: $F(2, 15) = 0.0081$, $p = .9919$; dark cycle, $F(2, 15) = 0.2790$, $p = .7604$). We did observe an elevation in VO$_2$ over time that in the dark cycle that was independent of treatment (two-way RM ANOVA using time as the factor: $F(9, 135) = 2.833$, **$p = .0044$). Likewise, there was no difference in carbon dioxide production (VCO$_2$) with respect to any treatment (Figure S3; two-way RM ANOVA using treatment as the factor: light cycle: $F(2, 15) = 0.4245$, $p = .6617$ dark cycle, $F(2, 15) = 1.035$, $p = .3793$). Interestingly, we observed a treatment effect with a measured reduction in the respiratory exchange ratio (RER) that was restricted to the light cycle (Figure 8e–f). RER is the ratio of the volume of CO$_2$ produced to the volume of O$_2$ consumed (VCO$_2$/VO$_2$) and is an indicator of which nutrients are being metabolized for energy. RER is 0.7 when lipids are being metabolized, 0.8 for proteins, and 1.0 for carbohydrates (Ramos-Jimenez et al., 2008). When comparing changes in RER during the light cycle (+2 to +12), there was a significant reduction in RER for animals receiving HisMgTx compared to PBS (Figure 8e, two-way RM ANOVA using with treatment as the factor: $F(2,14) = 4.677$, *$p = .0278$). There was not a significant effect for QDMgTx or a time × treatment interaction, $p = .0893$. Figure 8g illustrates the mean RER for all mice (+6 to +7) to incorporate a visualization of the specific RER effect associated with the light cycle.

### 3.4.3 Inhibition of Kv1.3 in the OB of obese mice may change ingestive behavior without differentially affecting locomotor activity

To complement measurements of EE, VO$_2$, and RER, we measured changes in total calories consumed by mice or changes in their...
locomotor activity by comparing baseline values prior to and after drug delivery (−1 versus. +11, Table 1). The total caloric intake and water consumption were not significantly different for mice in any drug treatment group (paired t test, p > .05). Despite eating the same amount of calories, mice that received either HisMgTx or QDMgTx reduced their number of feeding bouts while either increasing bout duration or increasing amount of food/bout (paired t test, p < .05), neither of which were observed in the PBS control mice. HisMgTx-treated mice decreased their number of feed bouts roughly 50% while increasing the feeding duration approximately 66%. QDMgTx-treated mice also decreased their number of feed bouts roughly 50%, but had no change in feeding duration while increasing food/bout by about 30%. All mice exhibited greater ambulatory locomotor activity in the dark cycle over that of the light cycle, as anticipated for a nocturnal animal. Although individual mice varied in their baseline ambulatory activity in the dark cycle (−1), all mice exhibited significantly reduced activity regardless of drug treatment, inferring less overall locomotor activity attributed to surgery or acclimation to the CLAMS extended environment (−1 versus. 11; Table 1, paired t-test, *p < .05, **p < .01).

4 | DISCUSSION

Herein, we produced a polyhistidine-modified peptide that inhibits Kv1.3 and can be self-assembled to fluorescent QDs through a one-step metal-histidine coordination. We demonstrated that this coupling strategy did not significantly alter the inhibitor interaction with the channel as determined by electrophysiological analyses, and it allowed sufficient conjugation to permit in vivo investigation of targeted delivery to a brain area that highly expresses Kv1.3. Most significantly, we discovered that osmotic pump administered HisMgTx and QDMgTx to the OB caused a loss of body weight in DIO mice and increased their ability to clear a glucose challenge. The loss in body weight was not attributed to a change in oxygen consumption or total energy expenditure. Treatment with the peptide inhibitor alone (HisMgTx) caused a reduction in RER in the light cycle, suggesting a shift in fuel utilization to fats over that of carbohydrates by blocking Kv1.3 conductance selectively in the OB. Interestingly, this did not cause a change in total caloric consumption, rather a change in ingestive behavior whereby mice significantly decreased feeding bouts while eating longer or larger meals.
Recombinant expression of the Kv1.3 inhibitor (HisMgTx) permitted conjugation to the Zn$^{2+}$ surface of QDs using polyhistidine-mediated self-assembly, while retaining a majority of predicted biophysical properties of the channel in a blocked state. Addition of a polyhistidine tag only slightly decreased the inhibitory potency of the peptide (IC$_{50}$ of 487 pM compared to reported IC$_{50}$ values.

**FIGURE 8** Inhibition of Kv1.3 in the olfactory bulb of obese mice reduces respiratory exchange ratio (RER) without changing energy expenditure (EE) and $V_{O_2}$. Normalized EE in the (a) light and (b) dark cycle, $V_{O_2}$ in the (c) light and (d) dark cycle, and RER in the (e) light and (f) dark cycle. Data represent mean ± SEM with number of animals per treatment group as noted. Notations and statistical analyses as in Figure 7b. (g) Mean RER of all mice over time across the light and dark cycle of days 6 and 7.
of 112–119 pM for unconjugated peptide (Garcia-Calvo et al., 1993; Knaus et al., 1995)). Despite this small reduction in potency, block of the channel occurred quickly. MgTx is amenable as a peptide blocker because of the fact that it has an extremely slow $k_{off}$ of 2 hr, and yet its $k_{off}$ is also rather slow for a vestibule blocker (Fadool & Levitan, 1998; Knaus et al., 1995). Conjugation of the inhibitor to the QD (QDMgTx) reduced the potency of channel inhibition ($IC_{50} = 894$ pM), however, the binding kinetics were equally as strong as that of HisMgTx, taking an average of 32 and 73 s to reach half block, respectively. Regardless of the measured inhibitory potency of either form of the inhibitor, both had rapid association with the channel and were equally effective blockers, causing a loss of 73%–85% of the outward current in Kv1.3-expressing HEK293 cells. When tested on ex vivo slices of the OB, QDMgTx, but not QDs alone, increased AP firing frequency by decreasing the pause duration between spike trains. Although we observed that QDMgTx binds non-specifically to mock-transfected HEK293 cells, functionally, we found that QDs did not enhance excitability of mitral cells, which is consistent with their inability to block Kv1.3 voltage-activated conductance in HEK293 cells. These results suggest that the conjugated HisMgTx, not the QD, is acting to increase mitral cell excitability.

It is not clear why QDMgTx was effective in labeling Kv1.3-expressing HEK293 cells but not mitral cells given the slow $k_{off}$ for the MgTx interaction with the channel. Given the confirmation of the accuracy of the cannula placement and the calculated volume of the OB with determined osmotic delivery rates, it is unlikely that the inhibitors traveled beyond the OB region. Moreover, dye tracking and viral infection at similar flow rates and injection volumes to the OB in our hands did not reveal labeling outside the OB. Certainly, catabolism or clearance of the QD is feasible given the duration of delivery, and would be a factor to reduce sustained visibility of the label in vivo. Previous studies have shown that QDs are cleared within hours or days, depending on route of delivery (Fischer et al., 2006; Medintz et al., 2008). Likewise, a greater concentration of the conjugate may also be required for in vivo delivery to visualize QDs with fluorescence microscopy, while transmission electron or confocal microscopy, as others have used (Brunetti et al., 2018; Dante et al., 2017) may be a more appropriate technique for visualizing signal at low or therapeutic concentrations.

Immobilization of polyhistidine-containing biomolecules on the Zn$^{2+}$ surface of QDs has been widely applied and is an attractive route of conjugation (Clapp et al., 2004; Dif et al., 2009; Wang et al., 2016). We previously covalently conjugated MgTx to QDs by activating carboxyl groups on the designed QDs such that they would form an amide bond with basic residues found on the surface of MgTx (Schwartz et al., 2017). Although this method was not capable of producing sufficient yields to perform in vivo studies, it produced a conjugate that inhibited voltage-activated Kv1.3 currents (66% inhibition) in HEK293 cells and was selective to Kv1.2 and 1.3 Shaker family members when screened for selectivity (Kv1.1 to 1.7). Although we did not retest for selectivity with our newly synthesized HisMgTx and QDMgTx conjugate, herein, we did not expect the selectivity to be different than the native MgTx peptide because the primary surface interaction of the peptide has been identified via NMR and mutational and molecular dynamic studies to be on the peptides three β-sheets (Johnson et al., 1994; Khabiri et al., 2011; Nikouee et al., 2012; Yu et al., 2004) that we did not modify.

Having now produced therapeutic quantities of HisMgTx and QDMgTx, we were interested if pharmacological inhibition of Kv1.3 in the OB could alter metabolic function in DIO mice. Previous work has shown that pharmacological inhibition of Kv1.3 or whole-body

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**TABLE 1** Locomotor and Feeding behavior of obese mice treated with PBS, HisMgTx, or QDMgTx via cannulated osmotic mini-pump

<table>
<thead>
<tr>
<th></th>
<th>PBS Day −1</th>
<th>PBS Day 11</th>
<th>HisMgTx Day −1</th>
<th>HisMgTx Day 11</th>
<th>QDMgTx Day −1</th>
<th>QDMgTx Day 11</th>
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<tr>
<td>X-Axis Beam Breaks</td>
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<tr>
<td>12h Light (Cumulative)</td>
<td>4.656 ± 3.037 (6)</td>
<td>1552 ± 342</td>
<td>6.783 ± 2.403 (7)</td>
<td>2.667 ± 1.296</td>
<td>4.590 ± 2.205 (5)</td>
<td>1.216 ± 551</td>
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<tr>
<td>12h Dark (Cumulative)</td>
<td>12.495 ± 2056 (6)</td>
<td>6.892 ± 1627$^{\ast}$</td>
<td>25.376 ± 4.464 (7)</td>
<td>8.649 ± 3.073$^{\ast\ast}$</td>
<td>14.935 ± 1993 (5)</td>
<td>5.376 ± 466$^{\ast}$</td>
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<td>Water Intake (g)</td>
<td>2.6 ± 0.3 (5)</td>
<td>3.1 ± 0.3</td>
<td>3.3 ± 0.3 (7)</td>
<td>3.8 ± 0.5</td>
<td>3.6 ± 0.5 (4)</td>
<td>3.2 ± 0.2</td>
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<td>Caloric Intake (kcal)</td>
<td>13.9 ± 1.2 (6)</td>
<td>14.6 ± 0.3</td>
<td>14.7 ± 1.0 (7)</td>
<td>12.6 ± 1.5</td>
<td>16.0 ± 1.2 (5)</td>
<td>15.1 ± 0.9</td>
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<td>Feeding Bouts</td>
<td>21.3 ± 1.9 (6)</td>
<td>18.5 ± 2.0</td>
<td>37.3 ± 4.1 (7)</td>
<td>20.0 ± 2.3$^{\ast}$</td>
<td>44.6 ± 7.0 (5)</td>
<td>18.8 ± 3.2$^{\ast}$</td>
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<td>Grams/Feeding Bout</td>
<td>0.2 ± 0.01 (6)</td>
<td>0.2 ± 0.01</td>
<td>0.15 ± 0.01 (7)</td>
<td>0.17 ± 0.02</td>
<td>0.17 ± 0.02 (5)</td>
<td>0.25 ± 0.01$^{\ast}$</td>
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<tr>
<td>Feeding Duration (s)</td>
<td>177.4 ± 65.4 (6)</td>
<td>256.8 ± 60.4</td>
<td>133.6 ± 24.1 (7)</td>
<td>222.5 ± 42.9$^{\ast}$</td>
<td>242.9 ± 60.6 (5)</td>
<td>320.3 ± 39.8</td>
</tr>
</tbody>
</table>

All values ± standard error of mean (SEM). Paired t-test, Day −1 versus Day 11.

$^{*}$p = .05.

$^{**}$p < .01.
deletion of the channel can significantly alter metabolism in mice. In the periphery, Kv1.3 is expressed in T-lymphocytes, brown fat, and white adipose tissue, whereas in the CNS, Kv1.3 is found in the dentate gyrus of the hippocampus, mitral cells in the OB, and in pyramidal cells of the pyriform cortex (Kues & Wunder, 1992; Fadool & Levitan, 1998; Marks & Fadool, 2007; Colley et al., 2009; Upadhyay et al., 2013; Al Koborssy et al. 2018). Whether there are differential mechanisms to reduce body weight by inhibiting Kv1.3 peripherally versus centrally is not known. When the Kv1.3 inhibitor ShK-186 was delivered to the periphery via intraperitoneal injection every other day over a 10-week period, DIO mice had improved glucose clearance and a reduction in body weight that was attributed to an activation of brown fat that increased β-oxidation of fatty acids. Mice had enhanced VO₂ and EE without a change in total caloric consumption or locomotor activity (Upadhyay et al., 2013). Upadhyay et al. (2013) reported that a peripherally administered Kv1.3 inhibitor increased RER, selectively in the light cycle, which in turn increased brown adipose tissue metabolism that may or may not have an effect on thermogenesis in humans (Carpentier et al., 2018). Although it is not clear if peripherally administered Kv1.3 blockers cross the blood-brain barrier, especially at the level of the olfactory system that is known to be leaky (Palouzier-Paulignan et al., 2012), our data demonstrate that direct application of Kv1.3 inhibitors to the OB reduces body weight in DIO mice by oppositely changing fuel metabolism (decreasing RER), improving glucose clearance, and altering ingestive behavior, rather than modifying VO₂ or EE. Our measured decrease in RER indicates an increased lipid oxidation over that of carbohydrates, but the mechanism of how this might be activated by the OB is unresolved.

In addition to increased metabolism, Kv1.3⁻/⁻ mice also have elevated olfactory ability, in terms of both odor discrimination and odor threshold (as to super-smeller mice) (Fadool et al., 2004; Thiebaud et al., 2014; Tucker et al., 2012; Tucker et al., 2008; Tucker et al., 2012; Xu et al., 2003). Kv1.3⁻/⁻ mice have smaller, supernumerary glomeruli, increased density of odorant receptors, and can discriminate odor molecules that differ by only one carbon atom (Biju et al., 2008; Fadool et al., 2004). While super-smell mice do not have changed caloric intake, they have irregular ingestive behaviors (increased meal frequency and decreased water bolus) and elevated locomotor activity (Fadool et al., 2004). Interestingly, genetic ablation of olfactory sensory neurons, resulting in a loss of olfactory ability, was shown to selectively reduce body weight in DIO but not control fed mice (Riera et al., 2017). The reduction in body weight occurred without a change in caloric intake but rather a significant increase in VO₂ and EE in both the light and dark cycle. The reduction in body weight was thought to be caused by an activation of white and brown fat to increase lipolysis. A recent study (Mutlu et al., 2020) identified select olfactory neurons in C. elegans that could regulate fat metabolism without altering ingestive behavior. Inhibition of neuropeptide olfactory circuits in the worm revealed a molecular mechanism using glucocorticoid-inducible kinase to regulate fat metabolism in an odor-specific fashion. This further suggests that olfactory physiology can influence metabolic function.

Nutritional status is well understood to influence olfactory physiology. In the OB, Kv1.3 is expressed in mitral cells, the primary projection neurons of the OB that project to higher regions of the brain via the lateral olfactory tract. Mitral cell activity is primarily stimulated by olfactory sensory input, however, feeding state and nutritional status play a role as well (Aime et al., 2014; Thiebaud et al., 2014). Glucagon-like peptide 1 (GLP-1), insulin, and glucose all increase the excitability of mitral cells by reducing Kv1.3 activity (Fadool & Levitan, 1998; Fadool et al., 2000; Tucker et al., 2010; Tucker et al. 2013; Thiebaud et al., 2016). In obesity, GLP-1 secretions are known to be reduced from sources outside of the OB (Faerch et al., 2015; Ranganath et al., 1996) and the ability to clear glucose is limited (Thiebaud et al., 2014). Likewise, a concurrent reduction in olfactory ability is observed (Thiebaud et al., 2014). At the level of the OB, the ability of insulin to increase mitral cell activity is significantly reduced (Fadool et al., 2011) following diet-induced obesity. An altered action of GLP-1, insulin, and glucose on Kv1.3 in obese mice, brought on by altered levels or signaling, could lead to changed excitability of the output neurons to higher olfactory cortical and hypothalamic regions to change metabolic set-point.

Herein, we delivered HisMgTx/QDMgTx to the OB in obese mice, essentially jumpstarting a pathway dampened by nutritional state, and observed a reduction in body weight and improved glucose clearance, and with HisMgTx, a significant reduction in RER. RER is an indicator of which nutrients are being metabolized for energy and a reduction suggests a shift in fuel utilization to fats rather than carbohydrates. This identifies for the first time a unique role of the OB in energy regulation. Interestingly, central infusions of GLP-1 have similar impacts on energy regulation. GLP-1R activation in the CNS has been shown to reduce RER (Hwa et al., 1998; Kooijman et al., 2015), improve glucose clearance (Kooijman et al., 2015; Sandoval et al., 2008a, 2008b) and reduce caloric intake. Here, we observed an effect on ingestive behavior owing to HisMgTx and QDMgTx delivery to the OB, however, caloric intake was difficult to monitor because of the cannulated surgery having a significant influence on this variable. Likewise, we previously identified the unique presence of GLP-1-secreting cells known as preproglucagon neurons in the OB, however, it is unclear if these cells act as a driver of energy regulation (Thiebaud et al., 2016, 2019). The exact mechanism by which neuromodulation of Kv1.3 influences metabolism and fuel utilization is still under exploration, but it is clear that direct inhibition of Kv1.3 in the OB has a significant effect on metabolic function.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST
The authors have no interests to declare, scientific or financial.

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REFERENCES
Dennis, A. M., Sotto, D. C., Mei, B. C., Medintz, I. L., Mattoussi, H., & Bao, G. (2010). Surface ligand effects on metal-affinity coordination


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