tTARGIT AAVs: A sensitive and flexible method to manipulate intersectional neuronal populations

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Summary

While Cre-dependent viral systems permit the manipulation of many neuron types, some cell populations cannot be targeted by a single DNA recombinase. Although the combined use of Flp and Cre recombinases can overcome this limitation, insufficient recombinase activity can reduce the efficacy of existing Cre+Flp-dependent viral systems. We developed a sensitive dual recombinase-activated viral approach: tTA-driven Recombinase-Guided Intersectional Targeting (tTARGIT) AAVs. tTARGIT AAVs utilize a Flp-dependent tetracycline transactivator (tTA) “Driver” AAV and a tetracycline response element (TRE)-driven, Cre-dependent “Payload” AAV to express the transgene of interest. We employed this system in *Slc17a6^{FlpO};Lepr^{Cre}* mice to manipulate LepRb neurons of the ventromedial hypothalamus (VMH; LepRb^{VMH} neurons) while omitting neighboring LepRb populations. We defined the circuitry of LepRb^{VMH} neurons and roles for these cells in the control of food intake and energy expenditure. Thus, the tTARGIT system mediates robust recombinase-sensitive transgene expression, permitting the precise manipulation of previously intractable neural populations.

Keywords: Ventromedial hypothalamus, leptin receptor, intersectional populations, energy expenditure, tetracycline transactivator

Introduction

The molecular heterogeneity of the nervous system requires a rich toolkit for precise study of distinct cell populations. Together with Cre recombinase-expressing mice, the available suite of Cre-dependent viral vectors permits the manipulation of genetically-identified neural types. However, this approach neither permits the study of subpopulations of cells expressing a particular Cre allele (Hodge et al., 2019; Mickelsen et al., 2019) nor the study of Cre-expressing cells within a defined CNS site while excluding the Cre-expressing cells in closely-opposed brain regions.

Restricting transgene expression to cells that express two marker genes can overcome this challenge (Luan and White, 2007). Early attempts at this approach utilized gene-specific, Cre-sensitive transgene-expressing alleles in combination with Cre alleles driven by distinct genes (Chen et al., 2011). Another solution involves expressing two Cre fragments across different transgenes with distinct promoters, reconstituting active Cre only in cells that express both pieces (Hirrlinger et al., 2009). These approaches generally involve substantial investments of time and resources, however, as they require generating and interbreeding multiple new gene- and experiment-specific alleles.

Transgene expression can also be directed to cell types defined by two marker genes through the use of recombinase-dependent alleles (often inserted into the *Rosa26* Locus) containing a ubiquitous promoter and tandem STOP cassettes that are each excised by distinct recombinases (Daigle et al., 2018; Sciolino et al., 2016).

Neuroscience research often requires a higher degree of spatial specificity than afforded by the intersectional genetic models outlined above. Stereotaxic injections of recombinase-sensitive viral vectors can restrict transgene expression to a narrow anatomical region. Adeno-associated viruses (AAVs) generally represent the preferred viral system for Cre-dependent transgene expression, given their minimal toxicity and the speed and ease of their generation. Developing AAVs sensitive to multiple recombinases has been challenging because of the limited AAV genome size, which precludes the use of multiple recombinase-sensitive STOP cassettes (Wu et al., 2010).

The INTRSECT system overcomes this limitation by utilizing a single AAV vector that flanks the transgene coding sequence with lox and FRT sites in such a way that combinatorial expression of Cre and Flp permits expression of a functional pre-mRNA that can then be spliced to produce a mature coding sequence (Fenno et al., 2020, 2014). The multiple inversion and splicing steps involved in this system can limit transgene expression, however, perhaps due to the relatively poor recombinase activity of Flp (and even optimized FlpO). Furthermore, generating INTRSECT viruses that express new transgenes requires a relatively complex and labor-intensive design and optimization process (Fenno et al., 2020, 2017).

Seeking a dual recombinase-activated AAV system to overcome these limitations and that could be modified quickly and easily, we generated tetracycline transactivator (tTA)-driven Recombinase-Guided Intersectional Targeting (tTARGIT) AAVs composed of a Flp-dependent tetracycline transactivator (tTA) “Driver” AAV and a tetracycline

response element (TRE)-driven Cre-dependent “Payload” AAV to express the transgene of interest.

We applied tTARGIT AAVs to the study of ventromedial hypothalamic LepRb (VMH; LepRb^{VMH}) neurons, which modulate metabolic adaptations to obesogenic diets (Bingham et al., 2008; Dhillon et al., 2006) but have proven difficult to study directly due to the density and proximity of neighboring LepRb populations. Together with *Lepr^{Cre}* and *Slc16a7^{FlpO}*, tTARGIT AAVs allowed us to overcome these challenges and reveal a specific role for LepRb^{VMH} neurons in suppressing food intake and increasing energy expenditure to promote weight loss.

Results

The density of LepRb neurons in the adjacent arcuate nucleus (ARC), dorsomedial hypothalamus (DMH), and lateral hypothalamic area (LHA) complicated our initial attempts to study LepRb^{VMH} neurons using *Lepr^{Cre}* mice and Cre-dependent vectors; viruses targeted to the VMH spread to other nearby *Lepr^{Cre}* neurons, confounding the interpretation of our results (data not shown). Because *Slc17a6*, encoding the vesicular glutamate transporter 2 (vGLUT2) protein, expression is largely restricted to LepRb^{VMH} neurons and absent from most surrounding LepRb cells (Vong et al., 2011), we generated a *Slc17a6^{FlpO}* strain and crossed it with *Lepr^{Cre}* and a novel Flp- and Cre-dependent reporter (*Rosa26^{RCFL-eGFP-L10a}*). We then tested the potential ability of this combination of Cre and Flp alleles to specify LepRb^{VMH} neurons in the mediobasal hypothalamus. Although these reporter mice identified Cre- and Flp-co-expressing LepRb^{Slc17a6} neurons elsewhere in the brain, within the mediobasal hypothalamus this approach largely limited eGFP expression to VMH cells (Supplemental Figure 1).

We thus sought to use Flp- and Cre-dependent AAVs to target LepRb^{VMH} neurons and omit manipulation of non-VMH LepRb^{Slc17a6} cells. We injected the INTRSECT AAV system (Fenno et al., 2014) into the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* mice in an attempt to express channelrhodopsin (ChR2) in LepRb^{VMH} cells. However, this approach resulted in detectable ChR2 expression in one or fewer cells per section (Supplemental Figure 2). We surmised that while INTRSECT works well in systems with robust Flp and Cre activities, the poor recombinase activity of Flp and the more

moderate Flp and Cre expression mediated by *Slc17a6^{FlpO}* and *Lepr^{Cre}*, respectively, might limit INTRSECT-mediated transgene expression in LepRb^{VMH} cells.

We therefore set out to develop a more sensitive AAV system to drive Cre+Flp-dependent transgene expression, using as our framework a previously-described inducible gene expression system based on recombinase-dependent expression of the tetracycline transactivator (tTA) in combination with a tetracycline response element (TRE)-driven transgene-expressing allele (Chan et al., 2017; He et al., 2016). We packaged this system into two viral vectors, hereafter “tTA-driven Recombinase-Guided Intersectional Targeting” (tTARGIT) AAVs.

Our tTARGIT system utilizes “Driver” and “Payload” AAVs. The Driver (AAV-hSyn-Flex(FRT)-tTA) utilizes a Flex(FRT) cassette (Fenno et al., 2014) to Flp-dependently invert tTA, permitting its expression by a human synapsin I (hSYN1) promoter. This virus also contains two tetracycline operators (TetO) to drive a positive feedback loop and increase tTA expression (Chan et al., 2017). The Payload AAV mediates tTA/TRE-dependent transgene expression following its Cre-mediated inversion into the sense orientation. Hence, only cells that contain both recombinases express the transgene (Figure 1a). Tetracycline inhibits tTA-dependent gene expression (T. Das et al., 2016), so this system mediates constitutive payload expression in target cells in the absence of tetracycline.

To test the recombinase-dependence of this system, we combined the Flp-dependent Driver AAV and a Payload AAV that permits the tTA/TRE-mediated Cre-dependent expression of a ChR2-TdTomato fusion protein (ChR2-TdT; AAV-TRE-

Flex(Lox)-Chr2-TdT). We co-injected these viruses into the VMH of wild-type mice, mice that expressed either *Slc17a6^{FlpO}* or *Lepr^{Cre}* only, or *Slc17a6^{FlpO};Lepr^{Cre}* mice (Figure 1b, c). We detected no TdT (DSRed-immunoreactivity (-IR)) in the VMH of wildtype or *Slc17a6^{FlpO}* animals and minimal expression in *Lepr^{Cre}* mice. In contrast, the Driver+Payload combination mediated robust DSRed-IR in the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* mice (Figure 1c); furthermore, VMH photostimulation in these mice promoted robust colocalization of DSRed- and FOS-IR, consistent with the ability of this system to activate transduced Flp+Cre-expressing cells (Supplemental Figure 3). Importantly, DSRed-IR was restricted to the VMH in these mice.

We also tested the requirement for both the Driver and Payload viruses in this system by injecting each virus alone, or both viruses together, into the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* animals (Figure 1d). As expected, injecting either virus alone produced minimal or no detectable DSRed-IR, while coinjection of the two viruses yielded robust DSRed-IR (Figure 1e). Thus, robust transgene expression by the tTARGIT system requires injection of both the Driver and Payload AAVs, as well as the presence of both Flp and Cre recombinases.

The tTARGIT approach can also be modified from a Flp-ON/Cre-ON system, requiring both Flp and Cre recombinases for payload expression, to a Flp-ON/Cre-OFF system, mediating transgene expression in all Flp-expressing cells that do not contain Cre (Supplemental Figure 4). Placing the payload transgene in the forward orientation within the Flex(Lox) cassette permits tTA-driven transgene expression in all Flp-expressing cells that do not contain Cre, because Cre inverts the Payload transgene

into the antisense orientation. We tested this system with a novel Cre-inactivated Payload virus expressing a hM3Dq designer receptor exclusively activated by designer drugs (DREADD)-mCherry transgene. We coinjected this Cre-OFF Payload and the Flp-dependent Driver AAV into the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* animals on the Cre-dependent *Rosa26^{LSL-GFP-L10a}* background (Supplemental Figure 4b). As expected, this modified tTARGIT system drove hM3Dq-mCherry expression almost exclusively in cells that did not express the Cre-dependent GFP (Supplemental Figure 4c). We surmise that the few GFP-IR neurons with detectable mCherry might result from the low Cre expression mediated by *Lepr^{Cre}* (Patterson et al., 2011) and predict that the Flp-On, Cre-Off tTARGIT system should demonstrate complete Payload inactivation when used in conjunction with a more robustly-expressing Cre allele.

To define the projection targets of LepRb^{VMH} neurons, we developed a payload virus (AAV-TRE-Flex(Lox)-GFP-2A-SynmRuby) that encodes GFP plus a cotranslationally-expressed synaptophysin-mRuby transgene (Figure 2a). Coinjection of this tTARGIT Payload AAV and the Driver virus into the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* mice promoted robust VMH-restricted GFP-IR (Figure 2b). mRuby detection (DSRed-IR) for this virus was much lower than for GFP, however (data not shown); thus, we used GFP-IR to detect projections from LepRb^{VMH} cells. Assessing the entire CNS for the presence of GFP-IR revealed terminals in the periaqueductal gray (PAG), the arcuate, the periventricular thalamic nucleus (PVT), the periventricular hypothalamic nucleus (PVN), the bed nucleus of the stria terminalis (BNST) and the preoptic area

(POA) (Figure 2c-d). These are consistent with the known projections of the VMH (Canteras et al., 1994; Meek et al., 2016; Zhang et al., 2020).

VMH-specific (*Nr5a1*^{Cre}-mediated) *Lepr* ablation promotes obesity associated with decreased energy expenditure in high fat diet-fed animals, suggesting a specific role for LepRb in LepRb^{VMH} cells in the control of energy balance via the dietary modulation of energy utilization (Bingham et al., 2008; Dhillon et al., 2006). To define the function of LepRb^{VMH} cells, rather than the function of LepRb in these cells, we developed a Payload virus containing an inverted hM3Dq-mCherry transgene. We coinjected the Driver AAV and this AAV-TRE-Flex(Lox)-hM3Dq-mCherry Payload virus into the VMH of *Slc17a6*^{FlpO};*Lepr*^{Cre} animals (LepRb^{VMH-Dq} mice; Figure 3a). This approach promoted robust VMH-restricted expression of functional hM3Dq-mCherry as administration of the DREADD activator (Pei et al., 2008) clozapine-N-oxide (CNO) stimulated colocalization of FOS with DSRed-IR cells (Figure 3b-d).

To determine the potential modulation of energy expenditure, activity, and food intake by LepRb^{VMH} neuron activation, we placed LepRb^{VMH-Dq} animals in metabolic cages and administered either vehicle or CNO twice daily (Figure 3e-j). Compared to vehicle administration, activating LepRb^{VMH} neurons significantly increased 24-hour oxygen consumption (VO₂) and energy expenditure, both primarily due to effects during the light phase, despite decreasing ambulatory activity over 24 hours (primarily due to effects during the dark phase)(Figure 3g-h). Additionally, the hM3Dq-mediated activation of LepRb^{VMH} neurons also suppressed 24-hour food intake, primarily due to decreased light-phase feeding, revealing a previously unsuspected role for these cells

in the suppression of feeding. CNO also decreased the respiratory exchange ratio (RER) during the light phase, consistent with the increased metabolism of fat stores due to the combination of increased energy expenditure and decreased food intake.

To understand whether effects on brown adipose tissue (BAT) might contribute to the increased energy expenditure during LepRb^{VMH} neuron activation, we placed temperature probes in the interscapular space of LepRb^{VMH-Dq} animals to monitor BAT thermogenesis. Compared to controls, CNO significantly increased intrascapular temperatures in LepRb^{VMH-Dq} animals, suggesting LepRb^{VMH} neurons promote energy expenditure at least in part by augmenting BAT thermogenesis (Figure 3k-l).

As activating LepRb^{VMH} neurons increased energy expenditure and decreased food intake, we surmised that these neurons should promote weight loss. We thus administered CNO in drinking water to LepRb^{VMH-Dq} mice for three days. During this time, the body weight of LepRb^{VMH-Dq} mice decreased by approximately 10% (Figure 4a), returning to baseline following the cessation of CNO exposure. While CNO treatment decreased food consumption (largely during the second day of treatment) and water intake (Figure 4b-d). The magnitude and timing of these ingestive effects dictates that neither could account for the decreased body weight mediated by CNO, consistent with the notion that increased energy expenditure mediates the major effect of LepRb^{VMH} cells on body weight.

Hence, the use of our dual recombinase-dependent tTARGIT AAV system permitted us to determine that LepRb^{VMH} neuron activation increased energy

expenditure and decreased food intake during the inactive phase, suggesting the diurnal control of energy balance by LepRb^{VMH} neurons.

Discussion

The use of sequence-specific DNA recombinases (Cre, Flp and others) in conjunction with recombinase-dependent genetic alleles and viral vectors has revolutionized our ability to manipulate specific circuits and understand the central nervous system. The lack of robust viral systems to manipulate cell populations defined by the expression of multiple genes has impeded the study of more refined neural populations, however- including those identified by single cell RNA-sequencing (Campbell et al., 2017). Our tTARGIT AAV system addresses many shortcomings of previous intersectional tools, including limitations to transgene expression and the difficulty of incorporating novel transgenes into the AAV plasmids.

To facilitate the study of intersectional neural populations, we developed a suite of Cre-dependent tTARGIT Payload plasmids (Table 1). While we have not yet tested the function of all of these, our experience with the transgenes that we have tested predicts similarly robust expression of the various Payload AAV transgenes. The limitations of these Payload vectors are likely to mirror those of standard Cre-dependent viruses, including the requirement for stoichiometric transduction of/recombination in the cell type of interest to observe the effects of interfering with neuronal function. We have additionally generated Cre-inactivated Payload vectors to specifically mark Cre-negative Flp-expressing cells within the injection field; our preliminary findings suggest that these will work most effectively when used in conjunction with a robustly-expressing Cre allele.

While the tTARGIT AAV system as we have built it is designed to constitutively express transgenes in Cre- and Flp-expressing cells without the use of tetracycline-like compounds (usually doxycycline), it should be possible to decrease transgene expression from the tTARGIT AAVs by doxycycline treatment. Indeed, we built a Flp-dependent rtTA Driver virus that is predicted to require doxycycline treatment to mediate strong transgene expression. The rtTA-based system can drive low-level transcription independent of doxycycline (Zhu et al., 2001), however, and we have not yet tested this system.

The use of tTARGIT AAVs permitted us to target robust transgene expression to LepRb^{VMH} neurons specifically, in isolation from LepRb neurons in adjacent hypothalamic nuclei. While deleting *Lepr* from the VMH of chow-fed mice or restoring VMH *Lepr* expression on an otherwise LepRb-deficient background minimally (if at all) alters energy balance, knockout mice fail to increase energy expenditure on high-fat chow and become more obese than controls (Bingham et al., 2008; Dhillon et al., 2006; Gonçalves et al., 2014; Senn et al., 2019, p. 1). While these studies thus suggest a role for leptin action on LepRb^{VMH} cells in the control of energy expenditure, the manipulation of VMH *Lepr* expression does not otherwise permit the broader study of LepRb^{VMH} cells. In contrast, our use of the tTARGIT system, together with *Slc17a6^{FlpO};Lepr^{Cre}* mice, identified the projections of LepRb^{VMH} cells, demonstrated their ability to acutely suppress food intake as well as promoting energy expenditure (identifying BAT thermogenesis as a target for these cells), and revealed the diurnal nature of LepRb^{VMH} neuron-mediated control of energy balance. Presumably, the

finding that LepRb^{VMH} neuron activation alters food intake and energy expenditure specifically during the light cycle suggests that these neurons may decrease in activity during the inactive/light phase, permitting us to observe the effects of artificial neuron activation during this time.

In summary, we have developed a suite of dyad AAV vectors for the study of intersectional neural populations marked by the co-expression of Flp and Cre or marked by expression of Flp in the absence of Cre. This tTARGIT system yields robust dual recombinase-sensitive expression of the desired payload *in vivo*. With this approach, we defined the neural circuitry and functional capacity of LepRb^{VMH} neurons. These intersectional genetic tools will facilitate the study of a broad range of dual gene-defined cell populations across the central nervous system.

Materials and Methods

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martin Myers Jr (mgmyers@med.umich.edu).

Animals

Mice were bred in the Unit for Laboratory Animal Medicine at the University of Michigan. These mice and the procedures performed were approved by the University of Michigan Committee on the Use and Care of Animals and in accordance with Association for the Assessment and Approval of Laboratory Animal Care and National Institutes of Health guidelines. Unless otherwise indicated, mice were provided with *ad libitum* access to food (Purina Lab Diet 5001) and water in temperature-controlled (25°C) rooms on a 12 h light-dark cycle with daily health status checks. *Rosa26^{LSL-eGFP}*-*L10a* mice (Krashes et al., 2014) and *Lepr^{Cre}* mice (Leshan et al., 2006) have been described previously. Both male and female mice were used for all studies. Sample

Generation of *Slc17a6^{FlpO}* mouse line

Slc17a6^{FlpO} mice were generated using recombineering techniques as previously described (Balthasar et al., 2005). Briefly, the FlpO transgene (Addgene plasmid #13793) and a LoxP-flanked neomycin selection cassette were subcloned after an optimized internal ribosome entry sequence (IRES). The IRES-FlpO-neomycin cassette was then targeted 3bp downstream of the stop codon of *Slc17a6* in a bacterial artificial chromosome. The final targeting construct containing the *Slc17a6*-IRES-FlpO neomycin

cassette and 4kb of flanking genomic sequence on both sides was electroporated into ES cells followed by neomycin selection. Appropriately targeted clones were identified by quantitative PCR and confirmed by southern blot analysis. Targeted clones were expanded and injected into blastocysts by the University of Michigan Transgenic Core. Chimeric offspring were then bred to confirm germline transmission of the *Slc17a6*-IRES-Flpo allele; the neomycin selection cassette was removed by breeding to the E2A-Cre deleter strain (Jax stock #003724).

Generation of Cre+Flp-dependent *Rosa26*^{RCFL-eGFP-L10a} and Flp-dependent *Rosa26*^{RFL-eGFP-L10a} mice

The targeting vector was developed by the Allen Brain Institute and obtained from AddGene (plasmid #61577). The neomycin resistance cassette and tdTomato sequence were removed and replaced with the eGFP:L10a coding sequences. The plasmid was then microinjected by the University of Michigan Transgenic Core into fertilized oocytes with Cas9 protein and gRNAs targeting the *Rosa26* locus (CACCGACTCCAGTCTTTCTAGAAG and AAAGTCTTCTAGAAAGACTGGAGTC). Tail DNA from the resulting pups was screened with PCR for the presence and proper insertion of the targeting vector. The Flp-dependent *Rosa26*^{RFL-GFP-L10a} mouse was generated by germline deletion of the lox-stop-lox cassette.

Stereotaxic Surgery

Mice were anesthetized with isoflurane (2%) and mounted in a stereotaxic frame (Kopf). Using standard surgical techniques, 150nL of virus was injected bilaterally via a

glass micropipette attached to a microinjector (picospritzer II) targeting the VMH (AP - 1.3 mm; ML $\pm$ 0.25 mm, DV -5.55 mm, relative to bregma).

For DREADD studies, hit sites were verified by mCherry detection (DSRed-IR) following euthanasia. Any data from mice in which mCherry was not detected within the VMH or was detected in other hypothalamic nuclei were discarded. Data from mice with either unilateral or bilateral viral hits were included.

tTARGIT AAV plasmid generation

To generate the AAV-hSyn1-TetOx2-FLEX(FRT)-tTA, the tTA transgene was placed within a Flex(FRT) cassette (using Addgene plasmid #55641, a gift from Karl Deisseroth). The tTA sequence was then removed from pAAV-ihSyn1-tTA (a gift from Viviana Gradinaru (Addgene plasmid # 99120) and replaced with the Flex(FRT)-tTA sequence. Similarly, AAV-hSyn1TetOx2-Flex(FRT)-rtTA was generated by first placing the rtTA sequence (Using rtTA sequence from Addgene plasmid #102423, a gift from Kian Peng Koh) in a Flex(FRT) cassette (using Addgene plasmid #55641, a gift from Karl Deisseroth). The tTA sequence was then removed from pAAV-ihSyn1-tTA (a gift from Viviana Gradinaru (Addgene plasmid #99120) and replaced with the Flex(FRT)-rtTA sequence.

To generate the payload viruses, the GFP cassette was removed from AAV-TRE-Flex(Lox)-GFP (a gift from Hongkui Zeng (Addgene plasmid #65449)) and replaced with ChR2-TdTomato (From Addgene plasmid #18917; gift from Scott Sternson), hM3Dq-mCherry (From Addgene plasmid #44361; a gift from Bryan Roth), GFP-2A-SynmRuby (Addgene plasmid #71760, a gift from Liquan Luo), HA-Cas9

(Addgene plasmid #61592, a gift from Feng Zhang), eGFP-L10a (provided by DPO (Allison et al., 2015)), Caspase3-2A-TEVp (Addgene plasmid #45580, a gift from Nirao Shah), GCaMP6s (Addgene plasmid #100845, a gift from Douglas Kim), SwiChRca-TS-YFP (Addgene plasmid #55631, a gift from Karl Deisseroth), or TVA-mcherry+oG (a gift from Marco Tripodi (Ciabatti et al., 2017)).

Immunostaining

For control experiments presented in Figure 1, mice were euthanized three weeks following viral delivery. Hit sites were verified using a marker virus (AAV-CMV-Cas9-HA(18)).

Upon the completion of DREADD studies, mice were injected with CNO (1mg/kg), sacrificed two hours post-injection and then perfused with 10% formalin. Brains were then removed and post-fixed in 10% formalin for 24 hours, before being moved to 30% sucrose for 24 hours. Brains were then sectioned as 30 μ m thick free-floating sections. Immunohistochemical and immunofluorescent staining was performed using standard procedures using anti-FOS (1:1000, #2250, Cell Signaling Technology), GFP (1:1000, #1020, Aves Laboratories) and DSRred (1:1000, #632392, Clontech) antibodies. Images were collected on an Olympus BX51 microscope.

Indirect Calorimetry Studies

Mice were singly housed one week prior to indirect calorimetry studies. Mice were placed into metabolic (CLAMS) cages in the University of Michigan Mouse metabolic Phenotyping Center (UM-MMPC) and further equilibrated for 24 hours. Subsequently, mice were then injected twice daily (930AM & 5PM) with vehicle for two

days followed by an additional two days of twice daily (930AM & 5PM) CNO (1 mg/kg).

Data is presented as the average of the two saline days compared to the average of the two CNO days.

Effect of CNO on energy balance

Mice were singly housed for one week prior to study. Mice were given *ad libitum* access to standard drinking water for 48 hours. For the subsequent 72 hours, standard water was replaced by water containing CNO (2.5 mg/100mL) and 1% glucose (to make the CNO palatable). CNO-laced water was changed daily. For the final 48 hours, mice were returned to standard drinking water. Body weight, food mass and water levels were recorded daily.

Intrascapular temperature measurements

The UM-MMPC placed temperature transponders (IPTT-300 model with corresponding DAS-7007R reader, Bio Medic Data Systems) in the intrascapular subcutaneous tissue directly under the conjunction part of the butterfly-shaped BAT under isoflurane anesthesia. Mice were allowed to recover for 14 d before testing. One day prior to testing, ambient temperatures were increased from 22°C to 30°C. On the day of testing, mice were randomized to either CNO (1 mg/kg) or saline injections and temperatures were recorded at -10, 10, 20, 30, 45, 60, and 120 min relative to injection time. Following one week, the experiment was repeated and treatment conditions (vehicle or CNO) reversed.

Optogenetic stimulation

A single fiber-optic cannula (Doric Lenses) was implanted above the VMH (A/P: 1.3 mm, M/L: 0.25 mm, D/V: 5.0 mm) and affixed to the skull using Metabond (Fisher). After 3 weeks recovery from surgery, mice were then subjected to optical stimulation using 473 nm wavelength laser using 20 mW/mm² irradiance. Light pulses were delivered by 1 s of 20 Hz photo stimulation and 3 s resting with multiple repetitions for one hour.

Viral packaging

The INTERSECT pAAV-nEF Con/Fon hChR2(H134R)-EYFP plasmid (Fenno et al., 2014) was procured through Addgene (plasmid #55644). All rAAV viruses were made at the University of Michigan Vector Core using ultracentrifugation through an iodixanol gradient. rAAVs were washed 3 times with PBS using Amicon Ultra Centrifugal Filter Units (Millipore) and resuspended in PBS + 0.001% Pluronic F68. Titters were assessed by qPCR (all were $>1 \times 10^{13}$).

Statistical Analysis

All Data is displayed as mean +/- SEM. Replicate number is included in each figure legend. Statistical analysis was performed in either Graphpad Prism 8 or R using either t-tests or ANOVAs with Dunnet's post-hoc test or linear mixed model. $P < 0.05$ was considered significant.

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Author contributions

PVS, JW, AHA JNF, RL, and ACR researched and analyzed data. PVS, JW, AHA, CL, JNF, RL, DPO, PK, MGM and ACR designed experiments. All authors reviewed and edited the manuscript. MGM is the guarantor of the manuscript.

Conflict of Interest Statement: CL is an employee of Novo Nordisk A/S; the authors have no other competing interests relevant to this manuscript.

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Figure 1

b

- ① AAV-hSYN-Flex(FRT)-tTA
+
② AAV-TRE-Flex(Lox)-Chr2-TdT

c

d

- ① AAV-hSYN-Flex(FRT)-tTA
or
② AAV-TRE-Flex(Lox)-Chr2-TdT

e

Figure 2

Figure 3

Figure 4

Table 1

Driver Viruses	Notes
AAV-hSYN1-Flex(FRT)-tTA	Drives expression in the absence of DOX
AAV-hSYN1-Flex(FRT)-rtTA	Drives expression in the presence of DOX; not tested
Payload Viruses	
<u>Flp-ON/Cre-ON (Payload inverted)</u>	
AAV-TRE-Flex(Lox)-hM3Dq-mCherry	Tested
AAV-TRE-Flex(Lox)-ChR2-TdTomato	Tested
AAV-TRE-Flex(Lox)-Cas9	Not tested
AAV-TRE-Flex(Lox)-TVA-oG-mCherry	Not tested
AAV-TRE-Flex(Lox)-eGFP-2A-TetanusToxin	Not tested
AAV-TRE-Flex(Lox)-SwiChR-eYFP	Not tested
AAV-TRE-Flex(Lox)-eGFP-L10a	Not tested
AAV-TRE-Flex(Lox)-GCaMP6s	Not tested
AAV-TRE-Flex(Lox)-Cleaved Caspase-3	Not tested
AAV-TRE-Flex(Lox)-GFP-2A-SynmRuby	Tested; GFP expression permits tracing; poor SynmRuby expression
<u>Flp ON/Cre-OFF (Payload Sense orientation)</u>	
AAV-TRE-Flex(Lox)-hM3Dq-mCherry	Tested; may require strong Cre driver for complete inactivation in Cre neurons
AAV-TRE-Flex(Lox)-ChR2-TdTomato	
AAV-TRE-Flex(Lox)-eGFP-2A-TetanusToxin	

Figure Legends

Figure 1: tTARGIT AAVs: a dual virus system to target intersectional populations.

(a) The tTARGIT system employs the combination of “Driver” (AAV-hSYN-Flex(FRT)-tTA) and “Payload” (AAV-TRE-Flex(Lox)-Payload) AAVs. The Driver virus encodes a Flp-dependent tetracycline transactivator (tTA) under control of the human synapsin I (hSYN1) promoter and two tetracycline operators (TetO). The Payload virus encodes a Cre-dependent Payload transgene under control of the tetracycline response element (TRE). (b-c) Experimental scheme (b) and representative images (c) showing the detection of TdTomato (DSRed-IR, red) following the co-injection of AAV-hSYN-Flex(FRT)-tTA and AAV-TRE-Flex(Lox)-ChR2-TdT into the VMH of (from left to right, as labelled) wild-type (WT), *Slc17a6^{FlpO}*, *Lepr^{Cre}*, or *Slc17a6^{FlpO};Lepr^{Cre}* mice. (d-e) Experimental schematic (d) and representative images (e) showing the detection of TdTomato (DSRed-IR, red) following the injection of (from left to right, as labelled): (1) AAV-hSYN-Flex(FRT)-tTA, (2) AAV-TRE-Flex(Lox)-ChR2-TdT, or the two viruses combined (1+2) into the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* mice. Scale bars= 100 μ m.

Figure 2: Defining the downstream projections of LepRb^{VMH} neurons. (a)

Experimental schematic showing the injection of the Driver AAV with the Payload AAV encoding a GFP-2A-SynmRuby transgene into the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* animals. (b-c) Representative image of GFP-IR (green) showing viral transduction in the VMH (b) and projections (c) in the periaqueductal gray (PAG), arcuate nucleus (ARC), paraventricular hypothalamic nucleus (PVN), bed nucleus of the stria terminalis (BNST)

and preoptic area (POA). (d) Cartoon showing the projection targets of LepRb^{VMH} neurons.

Figure 3: Activation of LepRb^{VMH} neurons decreases food intake in addition to increasing BAT thermogenesis and energy expenditure. (a) Approach for activating LepRb^{VMH} neurons by co-injecting the AAV-hSYN-Flex(FRT)-tTA Driver and the AAV-TRE-Flex(Lox)-hM3Dq-mCherry Payload viruses into the VMH of *Slc17a6^{FlpO}; LepR^{Cre}* animals. (b-d) Representative images showing mCherry detection (DSRed-IR, red) and FOS-IR (cyan) in LepRb^{VMH-Dq} mice administered Vehicle (b) or CNO (1 mg/kg)(c-d). The right-hand panel of (c) shows a digital zoom of the boxed region in the left panel. Scale bars= 100 μ m. (e-j) Results from metabolic cage analysis of LepRb^{VMH-Dq} mice (n=6) treated with either vehicle (gray) or 1mg/kg CNO (red) at 9:30 and 16:30 (dotted lines). Lines in left panels denotes mean value; shading denotes SEM. Each animal was treated with vehicle for 2 days followed by CNO for 2 days to allow pairing. Bar graphs to the right show the average for each mouse at each time point across 24 hours and separated by time in light cycle (Day = light, Night = dark). (k, l) Changes to intrascapular temperatures over 120 minutes in LepRb^{VMH-Dq} mice (n=8) or GFP-injected (n=7) controls following vehicle (gray) or CNO (red; 1 mg/kg) administration at 30°C. (l) shows area under the curve (AUC) for each treatment condition in (k). For metabolic cage studies, statistical significance was determined using either a paired t-test (full-day data), or a linear mixed model for effects by time of day. For interscapular temperature

measurements, significance was determined by paired t-test. * $P < 0.05$, ** $P < 0.01$, ***
 $P < 0.001$

Figure 4: Chronically activating LepRb^{VMH} neurons promotes negative energy balance. (a-c) body weight, food intake and water intake of chow-fed (n=8; red lines) LepRb^{VMH-Dq} mice receiving two days normal drinking water (days 0-2), followed by three days exposure to CNO-laced drinking water (days 2-5), followed by an additional 2 days of normal drinking water. Onset and termination of CNO treatment are denoted by vertical dashed lines in each panel. Controls (n=5; black lines) were LepRb^{VMH-Dq} mice that did not receive CNO on days 2-5. (d) Comparisons between body weight (closed black circles) and food intake (grey squares) over time in LepRb^{VMH-Dq} animals. Data is presented as mean±SEM. Significance was determined by linear mixed model; *** $p < 0.001$

Supplemental Figure 1

Supplemental Figure 1: An intersectional approach for targeting LepRb^{VMH} neurons. Representative image of GFP-IR (black) in the (a) hypothalamus of adult *Lepr^{Cre}; Rosa26^{LSL-eGFP-L10a}* (*Lepr^{Cre}*; left panel), *Slc17a6^{FlpO}; Rosa26^{FSF-eGFP-L10a}* (*Slc17a6^{FlpO}*; middle panel) and *Slc17a6^{FlpO}; Lepr^{Cre}; Rosa26^{RCFL-eGFP-L10a}* (*Slc17a6^{FlpO}; Lepr^{Cre}*; right panel) mice. (b) GFP-IR (black) in the ventral premammillary nucleus (PMv), periaqueductal gray (PAG), and nucleus of the solitary tract (NTS) in *Slc17a6^{FlpO}; Lepr^{Cre}; Rosa26^{RCFL-eGFP-L10a}* animals. Scale bar= 100 μ m.

Supplemental Figure 2

a

Supplemental Figure 2: INTRSECT transgene expression in *Slc17a6*^{FlpO};*Lepr*^{Cre}-defined LepRb^{VMH} neurons. (a) schematic of unilateral injection strategy for INTERSECT virus and injection site marker into the VMH of *Slc17a6*^{FlpO};*Lepr*^{Cre} mice. Representative images showing mCherry-IR (red, left panels) and YFP-IR (green, right panels) in the VMH of three separate *Slc17a6*^{FlpO};*Lepr*^{Cre} mice co-injected with a Cre-dependent hM3Dq-mCherry AAV (an injection site marker) and INTERSECT Flp-ON/Cre-ON ChR2-eYFP AAV. Scale bar= 100 μ m.

Supplemental Figure 3

Supplemental Figure 3: FOS response to photostimulation of tTARGIT AAV-driven ChR2 production in *LepRb^{VMH}* cells. (a) Experimental schematic showing unilateral co-injection of the Flp-dependent tTARGIT Driver AAV and ChR2-TdTomato-expressing tTARGIT Payload AAV targeting the VMH of *Slc17a6^{FlpO};Lepr^{Cre}* mice, with optic fiber implantation. (b,c) Representative image of ChR2-TdTomato (Red) and FOS-IR (Cyan) following one-hour photostimulation; c represents a digital zoom on the boxed region in (b).

Supplemental Figure 4

a

b

c

Supplemental Figure 4: Design and validation of the tTARGIT AAV approach to Flp-ON/Cre-OFF genetics. (a) The tTARGIT Flp-ON/Cre-OFF system is composed of a Flp-dependent tTA Driver virus (1) with a Payload virus (2) in which the transgene lies in the forward orientation unless Cre mediates its inversion. (b-c) Schematic of Flp-ON/Cre-OFF viral injection strategy (b) and representative images of mCherry-IR (magenta) and GFP-IR (green) from three independent injections of the Flp-ON/Cre-OFF tTARGIT system using an hM3Dq-mCherry transgene into the VMH of *Slc17a6^{FlpO}; Lepr^{Cre}; Rosa26^{LSL-eGFP-L10a}* mice (which express GFP in all *Lepr^{Cre}* cells). Bottom images show zooms of the boxed regions in the top panels. White arrowheads indicate localization between GFP and mCherry. Scale bars= 100 μm.