Functional Characterization of All Missense Variants in *LEPR*, *PCSK1*, and *POMC* Genes Arising From Single-Nucleotide Variants

T-P-3078

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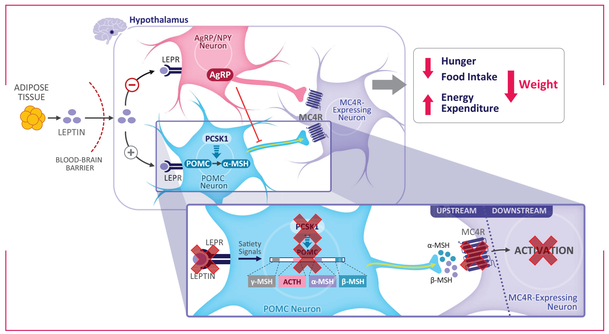
Summary

- To determine the functional effects of genetic variants on protein function and thus their potential contribution to MC4R pathway–related obesity, we performed functional characterization of all conceivable missense variants (arising from single-nucleotide variants [SNVs]) in leptin receptor (*LEPR*), proprotein convertase subtilisin/kexin type 1 (*PCSK1*), and proopiomelanocortin (*POMC*)
- Of all conceivable missense variants arising from SNV, 18.7% of *LEPR* variants, 45.4% of *PCSK1* variants, and 8.3% of *POMC* variants exhibited varying degrees of loss of function (LOF)

Introduction

- Rare genetic disorders of obesity are characterized by early-onset, severe obesity and hyperphagia[1]
- Rare genetic disorders of obesity can be caused by LOF variants in genes composing the melanocortin 4 receptor (MC4R) pathway, including *LEPR*, *PCSK1*, and *POMC*[1] (Figure 1)

Figure 1. The melanocortin 4 receptor pathway, a component of the central melanocortin pathway, helps regulate appetite, body weight, and energy expenditure, and loss-of-function variants in this pathway can cause rare genetic disorders of obesity.[1-4]



ACTH, adrenocorticotropic hormone; AgRP, agouti-related protein; LEPR, leptin receptor; MC4R, melanocortin 4 receptor; MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; PCSK1, proprotein convertase subtilisin/kexin type 1; POMC, proopiomelanocortin.

- To improve the understanding of the genetics underlying rare genetic disorders of obesity, it is crucial to characterize the impact of observed genetic variants on protein function

Objective

- To perform biochemical characterization and determine functionality of all conceivable missense variants arising from SNVs in *LEPR*, *PCSK1*, and *POMC*

Methods

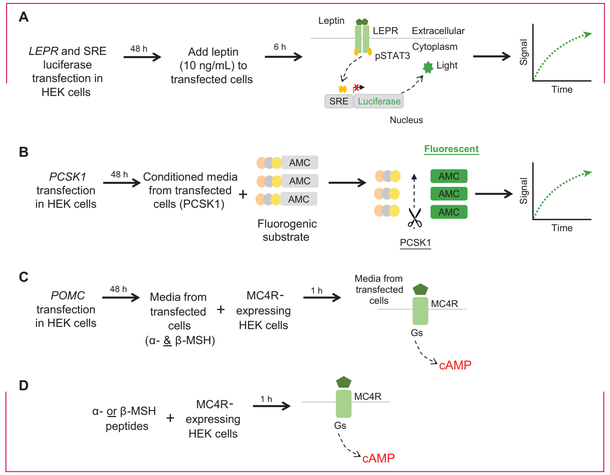
LEPR Assay[4]

- The functionality of *LEPR* variants was assessed using a LEPR-STAT3-luciferase assay, given that LEPR-STAT3 signaling is critical for leptin-regulated appetite and body weight (Figure 2A)
- Human embryonic kidney (HEK) cells were transiently transfected with relevant *LEPR* variants, firefly luciferase (LEPR-STAT3 responsive), and Renilla luciferase (transfection control)
- 48 hours after transfection, cells were serum starved for 6 hours and subsequently treated with leptin (10 ng/mL)
  - 6 hours after leptin treatment, the media were removed and the cell lysate was prepared using Dual-Glo® lysis buffer (Promega Corporation, Madison, WI)
  - The cell lysate was transferred to a 384-well plate and treated with Dual-Glo luciferase reagents according to the manufacturer's instructions
- Renilla and firefly luminescence were measured and the luminescence ratio was calculated on an EnVision plate reader (PerkinElmer Inc, Waltham, MA)

PCSK1 Assay[4]

- The functionality of *PCSK1* variants was investigated by measuring the processing of a fluorogenic substrate containing a PCSK1 cleavage site (Figure 2B)
- HEK293T cells were transiently transfected with *PCSK1* (wild-type [WT] or missense variants)
- To determine the PCSK1 catalytic activity, conditioned media from transfected cells were subjected to a fluorogenic assay
- PCSK1 activity was evaluated in triplicate in 50-µL reactions in a 96-well plate containing 25 µL of conditioned media and 25 µL of buffer containing final concentrations of 200 µM of substrate pyr-ERTKR-AMC, 100 mM of sodium acetate (pH 5.5), 2 mM of $CaCl_2$, 0.1% Brij 35, and 1× of protease inhibitor cocktail
- Reaction mixtures were incubated at 37°C and fluorescence measurements (380 nm excitation, 460 emission) were taken over 2 hours on an EnVision plate reader

Figure 2. Biochemical assays for **(A)** *LEPR*, **(B)** *PCSK1*, and **(C, D)** *POMC* variants.



AMC, 7-amino-4-methylcoumarin; cAMP, cyclic adenosine monophosphate; HEK, human embryonic kidney; LEPR, leptin receptor; MC4R, melanocortin 4 receptor; MSH, melanocyte-stimulating hormone; PCSK1, proprotein convertase subtilisin/kexin type 1; POMC, proopiomelanocortin; pSTAT3, phosphorylated signal transducer and activator of transcription 3; SRE, serum response element.

POMC Assay

- The functional effect of *POMC* variants was characterized using an MC4R–cyclic adenosine monophosphate (cAMP) assay[7]
- A transgene-based approach assessed the functional effect of *POMC* variants outside the α/β–MSH region (Figure 2C)
  - *POMC* variants were transfected in HEK cells
  - Media (α- and β-MSH) from transfected cells were collected 48 hours later and combined with MC4R-expressing HEK cells
  - After 1 hour, cAMP expression was quantified
- A peptide-based approach assessed the functional effect of *POMC* variants inside the α/β–MSH region (Figure 2D)
  - α- or β-MSH peptides for each missense variant (arising from SNV) were synthesized
  - Each α- or β-MSH peptide was combined with MC4R-expressing HEK cells
  - After 1 hour, cAMP expression was quantified

Variant Classification

- Variants were classified as significant LOF, moderate LOF, or WT-like on the basis of specific criteria (Table 1)
- Published variants in *LEPR*, *PCSK1*, and *POMC* underwent biochemical assays, the results of which were validated against the published functionality of each variant
- Every conceivable single missense variant in *LEPR*, *PCSK1*, and *POMC* was analyzed and functionally categorized
  - A subset of variants included those identified from genomic databases and previous publications

Table 1. Criteria for Variant Classification

Gene	Significant LOF	Moderate LOF	WT-like
LEPR	≤30% of WT	31%-70% of WT	≥71% of WT
PCSK1	≤30% of WT	31%-70% of WT	≥71% of WT
POMC (outside α/β–MSH)	E_{max} ≤30% of WT	E_{max} 31%-60% of WT	E_{max} ≥61% of WT
POMC (inside α/β–MSH)	E_{max} ≤30% of WT or EC_{50} >20x (WT EC_{50})	E_{max} 31%-60% of WT or EC_{50} (WT EC_{50} + 3SD) – (20 x [WT EC_{50}])	E_{max} ≥61% of WT and EC_{50} < (WT EC_{50} + 3SD)

EC_{50}, half-maximal effective concentration; E_{max}, maximum effect; LEPR, leptin receptor; LOF, loss of function; MSH, melanocyte-stimulating hormone; PCSK1, proprotein convertase subtilisin/kexin type 1; POMC, proopiomelanocortin; SD, standard deviation; WT, wild-type.

Results

Validation of Biochemical Assays Using Published Genetic Variants

- Results of each assay were largely consistent with previously published genetic variants (Table 2) in *LEPR*,[5-12] *PCSK1*,[13-19] and *POMC*[20-23]

Table 2. Validation of Assays Against Published Variants of Known Functional Impact

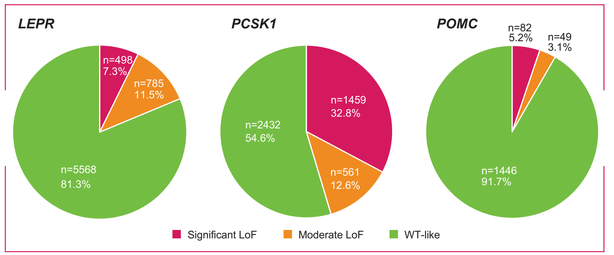
Gene	Variant	Published data[a]	Rhythm data (% of WT activity)	Validation[b]
LEPR	GFP_Control	NA	1.84	—
	W664R[8]	Significant LOF	Moderate LOF (53.21)	No
	A409E[8]	Significant LOF	Significant LOF (-3.78)	Yes
	H684P[8]	Significant LOF	Significant LOF (13.58)	Yes
	R612H[8]	Moderate LOF	WT-like (81.28)	No
	P878S[8]	Significant LOF	Significant LOF (-2.56)	Yes
	P876S[9]	Significant LOF	Significant LOF (-2.40)	Yes
	Q270P[10]	Significant LOF	Significant LOF (6.75)	Yes
	Y1141F[11]	Significant LOF	Significant LOF (-0.64)	Yes
	Q223R[12]	WT-like	WT-like (89.19)	Yes
PCSK1[c]	GFP_Control	NA	1.29	—
	G226R[13]	Significant LOF	Significant LOF (0.91)	Yes
	G209R[14]	Significant LOF	Significant LOF (1.84)	Yes
	N423K[14]	Significant LOF	Significant LOF (1.28)	Yes
	F548S[14]	Significant LOF	Significant LOF (1.57)	Yes
	G593R[16]	Significant LOF	Significant LOF (1.19)	Yes
	S307L[15]	Significant LOF	Significant LOF (2.09)	Yes
	N309K[16]	Significant LOF	Significant LOF (0.89)	Yes
	T175M[13]	Significant LOF	Significant LOF (6.66)	Yes
	M125I[13]	Significant LOF	Significant LOF (17.73)	Yes
	S24C[17]	WT-like	WT-like (78.34)	Yes
	S690T[18]	WT-like	WT-like (93.84)	Yes
	K26E[13]	WT-like	WT-like (84.97)	Yes
	H72L[19]	WT-like	WT-like (118.21)	Yes
	T558A[15]	WT-like	Moderate LOF (42.89)	No
POMC	GFP_Control	NA	1.30	—
	E105[23,d]	Significant LOF	0	Yes
	Y221C[20,e]	Moderate LOF	Moderate LOF (98.0, 173 nM)	Yes
	F144L[21]	Significant LOF	Significant LOF (23.56, >1 µM)	Yes
	D53G[24]	WT-like	WT-like (95.86)	Yes
	R236G[22,e]	Moderate LOF	WT-like (88.56)	No
	H143Q[20]	Significant LOF	Significant LOF (44.79, >1 µM)	Yes

LEPR, leptin receptor; LOF, loss of function; MSH, melanocyte-stimulating hormone; NA, not applicable; PCSK1, proprotein convertase subtilisin/kexin type 1; POMC, proopiomelanocortin; WT, wild-type. [a]Determined qualitatively on the basis of data in publication. [b]Determined by comparing percentage of WT activity category with published LOF category. [c]Wild-type activity measured at 1 hour. [d]Stop codon (nonsense mutation). [e]Mutation in β-MSH.

Functional Characterization of Missense Variants Arising From SNV in *LEPR*, *PCSK1*, and *POMC*

- Out of 6851 *LEPR* variants arising from SNV, 498 (7.3%) exhibited significant LOF, 785 (11.5%) exhibited moderate LOF, and 5568 (81.3%) exhibited WT-like activity (Figure 3A)
- Out of 4452 *PCSK1* variants arising from SNV, 1459 (32.8%) exhibited significant LOF, 561 (12.6%) exhibited moderate LOF, and 2432 (54.6%) exhibited WT-like activity (Figure 3B)
- Out of 1577 *POMC* variants arising from SNV, 82 (5.2%) exhibited significant LOF, 49 (3.1%) exhibited moderate LOF, and 1446 (91.7%) exhibited WT-like activity (Figure 3C)

Figure 3. Functional characterization of all missense variants arising from SNV in *LEPR*, *PCSK1*, and *POMC*.



LEPR, leptin receptor; LOF, loss of function; PCSK1, proprotein convertase subtilisin/kexin type 1; POMC, proopiomelanocortin; SNV, single-nucleotide variant; WT, wild-type.

Functional Characterization of Observed Missense Variants in *LEPR*, *PCSK1*, and *POMC*

- Out of 672 observed *LEPR* missense variants, 38 (5.7%) exhibited significant LOF, 98 (14.6%) exhibited moderate LOF, and 536 (79.8%) exhibited WT-like activity (Table 3)
- Out of 449 observed *PCSK1* missense variants, 138 (30.7%) exhibited significant LOF, 100 (22.3%) exhibited moderate LOF, and 211 (47.0%) exhibited WT-like activity
- Out of 233 observed *POMC* missense variants, 15 (6.4%) exhibited significant LOF, 9 (3.9%) exhibited moderate LOF, and 209 (89.7%) exhibited WT-like activity

Table 3. Functional Characterization of Observed[a] Missense Variants in *LEPR*, *PCSK1*, and *POMC*

	LEPR (n=672) n (%)	*PCSK1* (n=449) n (%)	*POMC* (n=233) n (%)
Significant LOF	38 (5.7)	138 (30.7)	15 (6.4)
Moderate LOF	98 (14.6)	100 (22.3)	9 (3.9)
WT-like	536 (79.8)	211 (47.0)	209 (89.7)

LEPR, leptin receptor; LOF, loss of function; PCSK1, proprotein convertase subtilisin/kexin type 1; POMC, proopiomelanocortin. [a]As reported in public databases (gnomAD, 1000 Genomes Project, DiscovEHR, NHLBI Exome Sequencing Project v. 6500) and scientific publications.

Acknowledgments: This study was sponsored by Rhythm Pharmaceuticals, Inc. Assistance with preparation of this poster was provided by Deirdre Rodeberg, PhD, MedThink SciCom, and funded by Rhythm Pharmaceuticals, Inc. The authors thank Lex H.T. Van der Ploeg for his assistance with data analysis and interpretation.

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