Commentary

MOLECULAR METABOLISM

Setting the TRAP for central leptin targets

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In this issue of Molecular Metabolism, Allison and her colleagues break new ground in our understanding of CNS leptin physiology [1]. By applying the sophisticated Translating Ribosome Affinity Purification (TRAP) technique in combination with RNA sequencing (TRAP-seq), they succeed in identifying novel markers for hypothalamic and brainstem subpopulations of leptin receptor (LepRb)-positive neurons with hitherto unknown function.

Allison and her colleagues crossed $Lepr^{Cre}$ mice with $Rosa26^{eGFP\text{-}L10a}$ mice to express the ribosomal eGFP-L10a fusion protein solely in LepRb-positive cells [1]. Anti-eGFP TRAP was subsequently used on homogenates of hypothalami or brain stems from several mice to pull down mRNA attached to ribosomes of LepRb-positive cells (TRAP-mRNA). RNA isolated from the TRAP-depleted homogenates (TRAP-depleted RNA) served as control. RNA sequencing and the subsequent comparative analysis of TRAP-mRNA vs. TRAP-depleted RNA revealed an enrichment of approx. 900 genes in brainstem LepRb cells and more than 1100 genes in hypothalamic LepRb cells. The presence of multiple genes known to be expressed in hypothalamic LepRb-positive neurons (e.g. Lepr, Agrp, Pomc) in the TRAP-mRNA fraction corroborated the overall TRAP-seq approach. Enrichment of marker genes for vasculature-associated cells (Flt1, Abcb1a, Tie1, Tek or Eltd1) was consistent with a role of LepRb in blood—brain-barrier function [2]. However, the nearly complete absence of marker genes for glial or immune cells in the TRAP-mRNA fraction was surprising given the role of leptin signaling in astrocytes [3] and suggests failure of LepRb-specific TRAP to recover mRNA from many non-neuronal cell types.

Overall, neuronal genes such as neuropeptides (Npw, Ucn, Prok2, Ghrh, Cartpt, Tac1) or marker genes for dopaminergic neurons (Slc6a3, Th) clearly prevailed in the hypothalamus and brainstem-derived mRNA profiles. Among the highly expressed and -enriched neuropeptide-encoding transcripts were prodynorphin (Pdyn), tachykinin-1 (Tac1), corticotrophin releasing hormone (Crh), and growth hormone releasing hormone (Ghrh) that have never been associated with LepR-positive cells and may serve as novel markers for unknown neuronal LepRb subpopulations.

TRAP-seq provides a unique opportunity to go beyond classical gene expression studies that — at best — reveal a blurred and heavily diluted snapshot of expression changes in a mixture of LepRb-positive and negative cells. LepRb-specific TRAP-seq overcomes those issues by solely purifying the translatome of LepRb-positive neurons. Nevertheless, a number of constraints for TRAP-seq remain. TRAP-seq doesn't allow discriminating between mRNA from cells actively expressing LepRb vs. cells that transiently expressed LepRb at an earlier time point. Accordingly, TRAP-seq will yield mRNA patterns from LepRb-positive cells, cells with temporally restricted LepRb expression during development, and cells with LepRb expression restricted to specific (patho-)physiological conditions. TRAP-seq also may be subject to inconsistent Cre-Lox recombination and incomplete excision of the transcriptional blocking cassette in the $ROSA^{eGFP\text{-}L10a}$ locus. Inconsistent or mosaic-patterned transgene expression would greatly impede subsequent TRAP-seq data. Such inconsistent Cre-lox recombination was shown for Pdyn in the arcuate nucleus of LepRb-neurons [1] and could result e.g. from insufficient LepR-driven Cre or Rosa26-driven eGFP-L10a transgene expression. Last, although TRAP-seq yields patterns of actively translated mRNA from all LepRb-positive cells, it does not discriminate between activated vs. silent LepRb cells. For all its shortcomings, TRAP-seq is a highly valuable tool that holds even more promise when combined with alternative methods. The closely related phosphorylated ribosome capture technique [4] allows for disentangling mRNA profiles of activated vs. silent neurons and further reveals expression changes in second order neurons activated by LepRb-positive cells. Single-cell sorting of LepRb-specific cell populations from distinct nuclei could further delineate the spatial distribution of leptin targets and reveal cell-to-cell heterogeneity and hidden subpopulations of cells [5]. Overall, the synergistic combination of methods and computational analyses will help sharpen the resolution of future experiments and identify novel cells and circuitry that mediate central leptin action. Needless to say, such synergism depends on open-access-repositories such as the Gene Expression Omnibus (GEO) project.

Allison and her colleagues restricted their TRAP-seq approach to the hypothalamus and brainstem, well known and important brain areas with dense LepRb expression that govern the homeostatic control of metabolism. Interestingly, only a relatively small number of marker genes were similar, pointing toward differential roles of LepRb in the two homeostatic brain areas. LepRb is also abundant in brain areas such as the thalamus, cerebellum, piriform cortex and hippocampus [6,7], and in hedonic control centers for ingestive behavior, such as the ventral tegmental area and substantia nigra of the midbrain [8]. Utilizing TRAP-seq on these brain areas with dense LepRb expression will further advance our understanding of hedonic and/or non-metabolic leptin action in the CNS.

In the hypothalamus, Allison and colleagues identified Pdyn as a novel marker for LepRb neurons. Expression of Pdyn was detected in the

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ventromedial (approx. 40% of all hypothalamic Pdyn-positive neurons) and dorsomedial hypothalamus (20%), in the arcuate nucleus (30%) and, to lesser extent, in the lateral hypothalamus and premammillary nucleus [1]. Male mice with Pdyn ablation in LepRb-positive cells displayed normal body weight, fat mass and food consumption when fed standard chow-diet. When challenged with a high fat diet, male $LepR^{Pdyn}$-null mice displayed a higher propensity for diet-induced obesity and elevated circulating leptin levels. Food intake was assessed for just 24 h and revealed no differences between genotypes. $LepR^{Pdyn}$-null mice displayed decreased energy expenditure (VO_2) compared to wild type littermates. Unfortunately, the data on oxygen consumption were derived from a rather small colony of 6–7 mice per genotype and normalized to body weight or lean mass. Such normalization is highly debated [9,10] and may lead to erroneous conclusions. Thus, ultimate answers on the role of Pdyn in LepRb positive neurons may require additional, larger cohorts of mice, more comprehensive monitoring of food intake, and ANCOVA analyses of energy expenditure to adequately account for covariates such as body weight, size, lean mass or fat mass. Overall, Pdyn seems to play a role in the regulation of body weight. However, its exact role in how leptin controls energy homeostasis remains elusive, and further research will be required to reveal the exact molecular role of Pdyn in CNS leptin action.

In summary, the study of Allison et al. [1] manages to disentangle novel genes and circuitry involved in hypothalamic and brain stem leptin action. Delineating cellular components of leptin signaling will ultimately facilitate the identification of novel, druggable CNS targets against obesity and type 2 diabetes. Whether Pdyn represents a druggable target remains to be determined. Allison and her colleagues nevertheless identified multiple other potential markers for LepRb subpopulations that remain unstudied. These markers are an actual gold mine for future research and may represent hidden treasures that help revealing the true nature of CNS leptin physiology.

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