Original Article

MOLECULAR METABOLISM

CART neurons in the arcuate nucleus and lateral hypothalamic area exert differential controls on energy homeostasis

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ABSTRACT

Objective: The cocaine- and amphetamine-regulated transcript (CART) codes for a pivotal neuropeptide important in the control of appetite and energy homeostasis. However, limited understanding exists for the defined effector sites underlying CART function, as discrepant effects of central CART administration have been reported.

Methods: By combining *Cart-cre* knock-in mice with a *Cart* adeno-associated viral vector designed using the flip-excision switch (AAV-FLEX) technology, specific reintroduction or overexpression of CART selectively in CART neurons in the arcuate nucleus (Arc) and lateral hypothalamic area (LHA), respectively, was achieved. The effects on energy homeostasis control were investigated.

Results: Here we show that CART neuron-specific reintroduction of CART into the Arc and LHA leads to distinct effects on energy homeostasis control. Specifically, CART reintroduction into the Arc of otherwise CART-deficient $Cart^{cre/cre}$ mice markedly decreased fat mass and body weight, whereas CART reintroduction into the LHA caused significant fat mass gain and lean mass loss, but overall unaltered body weight. The reduced adiposity in Arc^{CART};$Cart^{cre/cre}$ mice was associated with an increase in both energy expenditure and physical activity, along with significantly decreased *Npy* mRNA levels in the Arc but with no change in food consumption. Distinctively, the elevated fat mass in LHA^{CART};$Cart^{cre/cre}$ mice was accompanied by diminished insulin responsiveness and glucose tolerance, greater spontaneous food intake, and reduced energy expenditure, which is consistent with the observed decrease of brown adipose tissue temperature. This is also in line with significantly reduced tyrosine hydroxylase (*Th*) and notably increased corticotropin-releasing hormone (*Crh*) mRNA expressions in the paraventricular nucleus (PVN).

Conclusions: Taken together, these results identify catabolic and anabolic effects of CART in the Arc and LHA, respectively, demonstrating for the first time the distinct and region-specific functions of CART in controlling feeding and energy homeostasis.

Keywords CART; Energy homeostasis; AAV-FLEX; Arcuate nucleus; Lateral hypothalamic area

1. INTRODUCTION

The cocaine- and amphetamine-regulated transcript (CART) is a major neuropeptide involved in the regulation of diverse biological processes, including appetite control, maintenance of body weight, reward and addiction, psychostimulant effects, and neuroendocrine functions [1]. Among the wide and abundant central distribution of the peptide associated with multiple neurocircuitries in mammals [2–4], CART expression shows predominance in neuroendocrine neurons [5], particularly in the hypothalamus at feeding-related regions [6,7]. Extensive research has focused on the role of CART in modulating feeding behavior and energy homeostasis [1], thereby exploring therapeutic potentials in the treatment of obesity and other metabolic disorders. However, identification of the hypothalamic sites of action and the mechanisms underlying CART function in homeostatic regulation remains challenging, mainly owing to the lack of information on the elusive CART receptor(s) that remain(s) unidentified [1]. In addition to the ubiquitous expression and broad connection of the peptide with various neuronal networks, the biosynthesis of CART involves complex post-translational processing, including the formation of various disulfide bonds [5,8], further rendering pharmacological intervention using any synthetic *ex-vivo* produced CART analogs difficult.

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The functional importance of CART is highlighted by the strong evolutionary conservation across species [1,9]. Moreover, CART expression patterns are very similar between rodents and human, with high density of expression in hypothalamic areas important for energy homeostasis regulation, including the arcuate nucleus (Arc), lateral hypothalamic area (LHA), paraventricular nucleus (PVN), and dorsomedial hypothalamic nucleus [10,11]. However, studies of CART primarily conducted in rodents have so far generated inconclusive or discrepant results [1], highlighting the complexity in the physiology of endogenous CART biosynthesis and function. In rats and mice, in addition to alternative splicing of *Cart* mRNA on the transcription level, the resultant CART propeptides harbor several cleavage sites that are subjected to post-translational processing by prohormone convertases, which exhibit tissue- and brain region-specific expressions and functions [8,11,12]. Hence, the *in vivo* production of several CART isoforms also involves processing and expression in a tissue- and site-specific manner [8,12], generating at least two predicted active CART peptides, CART I (42-89/55-102) and CART II (49-89/62-102) [5,13]. Accordingly, studies in rodents have suggested the differential physiological functions of CART variants across the periphery as well as the central nervous system [8].

In light of the multiple factors involving complex region-specific post-translational processing, along with undetermined conformational profile and binding sites for the peptide, studies are challenged with limited targeting contacts to mimic or modulate endogenous CART expression and activity. Although most traditional studies have classified CART as anorexigenic [14–16], orexigenic evidences also exist [17–19]. In mouse models, global CART knockout led to an overall increase in body weight gain and adiposity, while lacking any overt impact on food consumption [1,20]. Supporting the catabolic property of CART, mutations and polymorphisms in the human *CART* gene are linked to obesity and associated metabolic disorders [21,22]. On the other hand, while intracerebroventricular (i.c.v.) administration of various CART isoforms in rodents consistently and dose-dependently suppressed food intake and weight gain [1,23], overexpression of CART I (55-102) in specific hypothalamic nuclei was shown to induce either stimulatory or inhibitory response on feeding and body weight [17,18,24]. This suggests that CART originating from different nuclei is engaged in differential functions involving discrete neurocircuitries. Furthermore, central administrations of different CART variants, which demonstrate varying endogenous abundance in distinct brain regions, have exhibited site-specific activities and differential potencies in affecting food intake and body weight gain [25,26].

In an effort to identify the most critical hypothalamic sites of CART action in the control of energy homeostasis, the Arc and LHA have received considerable interest. The Arc represents a pivotal structure for adiposity signal integration and metabolic modulation [27], where CART expression is enriched and colocalized with the anorexigenic proopiomelanocortin (POMC) [28,29]. The first-order CART-containing neurons at the Arc launch axonal projections to neurons at the LHA and PVN [30–32]. Despite implications for the Arc-LHA pathway in mediating the anorexic effects of CART [30,31], direct injection of CART into the Arc or LHA in rodents triggered overt orexigenic responses [17,18,24]. Differential neurochemical responses were also shown at the two regions. For instance, while *Cart* mRNA levels at the Arc are markedly reduced by fasting and restored following refeeding [33,34], such correlation was not demonstrated at the LHA [35]. Moreover, CART in the LHA is found to be colocalized with the orexigenic-acting melanin-concentrating hormone (MCH) [36,37], further hinting that CART in different hypothalamic areas and neurons may exhibit disparate functions.

In this study, we aimed to more specifically investigate the effects of CART in the Arc and LHA on the regulation of feeding and energy homeostasis. For this, we developed models that allow the CART neuron-specific introduction and processing of CART by combining *Cart-cre* knock-in mice with a *Cart* adeno-associated viral vector designed using the flip-excision switch (AAV-FLEX) technology [38]. The AAV vector encodes the long form of the mouse *Cart* cDNA CART I (55-102), which is focused herein due to wider documentation and the reported higher potency in influencing appetite and body weight compared to the CART II (62-102) isoform [25,39]. This approach allows for either reintroducing CART specifically into CART neurons in otherwise CART-deficient mice, or to overproducing CART in Arc or LHA CART neurons, avoiding off-target side-effects associated with simple ectopic delivery. Furthermore, our viral approach also facilitates the *in vivo* processing and modifications of CART propeptides in a natural physiological environment, hence producing active peptides under endogenous conditions. Mouse models generated this way were then analyzed in a comprehensive phenotyping paradigm, determining all major aspects that are involved in energy homeostasis control.

2. MATERIALS AND METHODS

2.1. Animals

All research and animal care procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Ethics Committee and were conducted in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Male mice were used for all experiments and were housed under conditions of controlled temperature (22 °C) and illumination (12:12 h light–dark cycle, lights on at 07:00 am). Mice were provided with *ad libitum* access to water and standard chow diet (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal/g; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia).

2.2. Generation of inducible *Cart-cre* knock-in mouse model

To investigate the effects of CART action at particular hypothalamic regions, CART neuron-specific introduction of genetic elements was performed in an adult-onset inducible manner. This is enabled through the use of a conditional *Cart-cre* knock-in mouse line, for which the detailed generating procedures were described previously [20]. As illustrated [20], the inducible *Cart-cre* knock-in mice ($Cart^{cre/cre}$) were generated by crossing the $Cart^{lox/lox}$ line with mice that expressed *Cre*-recombinase specifically in the oocytes to homozygosity, hence combining the tamoxifen-inducible *Cre* gene with the endogenous *Cart* promoter. In this study, both the homozygous $Cart^{cre/cre}$ and the heterozygous $Cart^{cre/+}$ mouse lines were included. Comparison between the two groups serves to highlight the contribution of CART function in specific hypothalamic nuclei to feeding and metabolic regulation in the presence or absence of endogenous CART signaling.

2.3. Generation and expression of cre-inducible *AAV-FLEX-Cart* vector

To selectively express the bioactive CART in CART neurons, the flip-excision switch technique was employed to create a unique *Cre*-recombinase-dependent AAV vector for delivery, which carries a double floxed inverted open reading frame (Figure 1). The FLEX strategy utilizes the recombination mechanism whereby DNA elements flanked by *loxP* recombination sites in the opposite orientation are inverted by Cre-recombinase [38]. The *AAV-FLEX-Cart* vector consists of a codon-optimized mouse *Cart* cDNA for the expression of CART I (55-102) active peptide flanked by *loxP*-sites as displayed in Figure 1A.

