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CART in the Regulation of Appetite and Energy Homeostasis

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Abstract

The cocaine- and amphetamine-regulated transcript (*CART*) has been the subject of significant interest for over a decade. Work to decipher the detailed mechanism of CART function has been hampered by the lack of specific pharmacological tools like antagonists and the absence of a specific CART receptor(s). However, extensive research has been devoted to elucidate the role of the CART peptide and it is now evident that CART is a key neurotransmitter and hormone involved in the regulation of diverse biological processes, including food intake, maintenance of body weight, reward and addiction, stress response, psychostimulant effects and endocrine functions[1,2]. In this review, we focus on recent knowledge gained on CART's role in controlling appetite and energy homeostasis.

1. Introduction

Experiments conducted with acute administration of cocaine or amphetamine in rodents resulted in the upregulation of a particular mRNA species in the striatum of the brain that was subsequently named 'cocaine- and amphetamine-regulated transcript' (CART) and the encoded peptides are referred to as CART peptides[3,4]. Importantly, CART mRNA levels were also found increased in the nucleus accumbens on post-mortem tissues from human victims of cocaine overdose[5]. CART is transcribed as two alternatively spliced mRNAs that are of different lengths and hence produce pro-peptides of different lengths, called proCART 1–89 and proCART 1–102[3,6]. However, the mRNA splicing has no effect on the final peptide, as the active parts of the CART peptides are encoded by a sequence that lies downstream of the spliced region and is therefore identical in both pro-peptides[3,7]. However, the proCART peptides contain several cleavage sites that allow post-translational processing by pro-hormone convertases in a tissue-specific manner[3,6-16]. This processing produces at least two known biologically active peptides, CART I (55–102) and CART II (62–102), each containing three potential disulphide bridges[3,7,17,18].

CART peptides are evolutionarily strongly conserved between rodent and human[6,19,20], with about 95% amino-acid identity between the active neuropeptides [6,17,18,21], suggestive of a conserved critical physiological function. Notably, high levels of CART expression have been identified to localize in brain regions that include the arcuate nucleus (Arc), the lateral hypothalamus area (LHA), the paraventricular nucleus (PVN) and the nucleus accumbens (Acb)[22,3,12,13,23-27], suggesting an important role for CART in the regulation of food intake and energy homeostasis. This is consistent with results from injections of CART peptide into the nucleus accumbens which have shown an inhibition of feeding in rodents[28,29]. In addition, CART in the Arc is co-localized with α-melanocyte stimulating hormone (α-MSH)[30,31], which is produced from the proopiomelanocortin (POMC) precursor and is a major inhibitor of appetite and food intake[32-34]. In the Arc, CART mRNA levels are regulated by circulating leptin [35,36] and are increased by peripheral leptin administration[37,35,38,39], again indicative of a critical role in energy balance regulation. Moreover, injection of CART I (55–102) into the PVN or Arc of rats markedly enhanced the mRNA expression for the uncoupling protein-1 (*UCP-1*) in brown adipose tissue, relating CART peptides not only to the control of feeding but also the modulation of energy expenditure[40,41]. Mechanistically, it has been shown that application of CART I (55–102) to hypothalamic explants can stimulate the release of corticotropin-releasing hormone (CRH) and thyrotropin releasing hormone, which further links CART to the regulation of the hypothalamic-pituitary-adrenal axis[42]. Importantly, several genetic studies have associated mutations or

polymorphisms in the *CART* gene in humans with obesity, clearly demonstrating a crucial role of CART in the control of energy homeostasis also in humans[43-50].
While a specific CART receptor(s) has not been identified to date, there is strong evidence that CART signalling can be blocked by pertussis toxin (PTX), indicative of the involvement of an inhibitory G-protein-coupling receptor that couples to $G_{i/o}$ proteins[21,51,52]. For example, CART I (55–102) has been described to inhibit voltage-gated L-type Ca^{2+} channels in hippocampal neurons, an effect that was blocked by treatment with PTX[21]. Furthermore, central administration of CART I (55–102) stimulated the phosphorylation of cyclic AMP-response-element-binding protein (CREB) in CRH neurons in the PVN of fasted and fed rats[53], which again is classified as a PTX-sensitive mechanism. Finally, CART I (55–102) application activated extracellular signal-regulated kinase (ERK) phosphorylation in the rodent pituitary-derived cell lines, AtT20 and GH3, and such CART-induced effects were attenuated by a MEK kinase inhibitor as well as pre-treatment with PTX[51], further supporting the mediation of CART action by inhibitory G proteins.

2. Distribution of CART expression

CART can be found in both the central nervous system (CNS)[3,4,12,17,23-25,54-56] and the periphery[12,57-60,61-64]. *CART* mRNA-containing neurons are present in high densities in diverse regions such as the Edinger-Westphal nucleus, ganglion cells in the retina, mitral and tufted cells of the olfactory bulb, sensory barrels in the cerebral cortex, the pituitary, lamina X of the spinal cord, medulla of the adrenal cortex, the vagal nuclei, and a number of hypothalamic nuclei[3,24]. Expression of CART in peripheral blood and pituitary portal has also led to the identification of CART-positive neuroendocrine neurons in the hypothalamus, where CART was demonstrated to constitute a releasing factor delivered to the hypothalamic-pituitary-adrenal (HPA) portal circuit for potential endocrine regulation[59,63]. Consistent with a role in energy homeostasis, CART expression has also been associated with glucose-sensing sites both centrally and peripherally, in both rodents and humans[64-68]. In the periphery, CART expression has been identified in the islet endocrine cells, ganglionic cells, as well as the sensory and autonomic nerve fibres of the pancreas[64-68]. A recent paper also reported the expression of CART mRNA and protein in subcutaneous and visceral white adipose tissues from both rat and human[69], serving a potential novel role in adipocytes by fine-tuning lipolysis and lipase activation to affect lipid- and glucose-homeostasis[69,70].

In addition to the structural conservancy between CART isoforms across species[3,6,20,71], functional conservation of CART in the mammalian neuroendocrine system has also been implicated in the resembling *CART* mRNA and peptide distribution pattern in the brain observed between humans, rodents, as well as monkeys[6,25,27,72]. As a leptin-regulated neurotransmitter with potent appetite-suppressing activity[26], CART expression in the CNS is highly localized to distinct brain areas critically involved in the control of energy homeostasis, limbic and sensory functions, as well as throughout the HPA axis[17,3,12,23,25,27,72]. Localization analyses of CART expressions at the mRNA and peptide levels have demonstrated concordance across studies applying cDNA sequencing[3], *in situ* hybridization[3,24] and immunohistochemistry[12]. Indeed, *CART* mRNA has been shown to constitute the third most abundant transcript identified in rat hypothalamus after subtraction of cerebellar and hippocampal mRNAs[25].

3. Central CART in the regulation of appetite and energy homeostasis

A considerable amount of information has been accumulated for the role of CART in modulating metabolism via actions within the CNS. Together with several other critical neuropeptides, CART is integrated into the circuits that control the overall regulation of energy balance. The major site of action is the hypothalamic arcuate nucleus located at the base of the hypothalamus in an area where

the blood-brain barrier is semi-permeable, hence uniquely assessable to circulating humoral and metabolic mediators[73-75]. As a consequence, the arcuate nucleus circuit serves as sensor of whole-body energy status represented by adiposity levels and promptly directs downstream responses accordingly through neuronal signalling to maintain a constant level of fuel stores[36,73].

Two sets of neurons with reciprocal metabolic effects reside in the Arc, namely the orexigenic neuropeptide Y (NPY)/AgRP neurons which promote food intake and reduce energy expenditure, and the adjacent anorexigenic POMC/CART neurons that inhibit food intake and increase energy expenditure[31,73,76]. In response to circulating hormones, such as leptin and insulin that are secreted from adipose tissues and the pancreas, respectively, with a plasma level proportionate to body adipose stores[77-79], hypothalamic expressions of the two sets of neurons are differentially regulated[36]. For instance, leptin and insulin levels are reduced following food deprivation that lowers body fat stores, which inhibit POMC/CART neurons and stimulate NPY/AgRP neurons[36]; the latter are also subject to activation by the circulating orexigenic peptide ghrelin released from the stomach with peak levels before meal initiation[80].

As opposed to two independent sensors mediating opposing effects and projecting parallel extensions to second-order hypothalamic neuropeptide neurons, intra-arcuate connections between the two neuronal subgroups have been depicted by neuroanatomical studies to comprise the autoregulation by melanocortin peptides as well as local γ-aminobutyric acid (GABA)-mediated effects[81,82]. POMC/CART neurons express the melanocortin 3-receptor (MC3-R) which is specific for melanocortin peptides derived from the post-translational cleavage of POMC[81], such as the anorectic α-MSH[32-34,83]. The activation of a subset of Arc POMC/CART neurons following exposure to leptin via leptin receptors has been reported to impose an autoinhibitory effect exerted through MC3-R activation in response to elevated release of POMC-derived peptides[38,82,84]. However, inconsistency exists with the proposed negative autoreceptor manner of MC3-R in diminishing POMC/CART neuronal activity due to the mild increase in adiposity and body weight observed in MC3-R-deficient mice[85,86]. In addition to the potential feedback mechanism described above, the inhibitory neurotransmitter GABA constitutes a principal modulator in the complex intra-arcuate circuit[82]. Subpopulations of Arc neurons harbor colocalized expressions of NPY and GABA[87], where GABA released from the orexigenic NPY/GABA terminals induces inhibitory postsynaptic currents (IPSCs) that consequently hyperpolarizing hence decreasing firing rate of the postsynaptic POMC/CART neurons[82]. Besides such direct neuronal innervations synapsing into Arc POMC/CART neurons by GABA-secreting NPY neurons[82], a recent investigation has demonstrated the direct action of leptin on hyperpolarizing presynaptic GABAergic neurons in reducing GABA-mediated inhibitory tone to POMC/CART cells[88], whilst ghrelin was shown to trigger the opposite events on the same neurons[89]. Collectively, the electrophysiological regulation of arcuate POMC/CART neurons constitutes two primary mechanisms, including the direct effects of potent neuropeptide modulators on membrane potential through influencing ion channel activity, as well as the indirect impacts on IPSC frequency marking GABA inhibition from local NPY/GABA neurons[82]. The resultant resting action potential is purported to concurrently involve the autoregulatory effects of melanocortin peptides [82], ultimately providing an integrated circuit at the Arc for the regulation of food intake and energy homeostasis.

Present in both classic neuroendocrine neurons and hypothalamic projection neurons, Arc CART displays an almost 100% colocalization with POMC and complete segregation from the more medially located orexigenic NPY in rodents[13,38,90,91]. The high levels of co-existence of CART with POMC and derivatives such as α-MSH were found to populate throughout the rostrocaudal extent of the Arc[13], wherein CART immunoreactivity (IR) was depicted in a separate study to pervade all α-MSH-IR perikarya and axons[92], which concords with the anorectic role widely adopted for POMC/CART neurons. In accordance with established neuronal projections from Arc[36], all α-MSH-IR axons in the PVN have also been shown to contain CART immunoreactivity[92].

In addition to co-storage with the POMC and associated cleavage products which promote negative energy balance[32-34,83], CART exhibits > 95 % colocalization with the orexigenic melanin-concentrating hormone (MCH)[93-95] at the LHA and zona incerta (ZI)[13,39,92]. All MCH-positive cells showed immunoreactivity for CART in the rostral ZI and the most medial region of the LHA, whilst an increasing number of non-CART MCH cells were detected in the caudal and lateral parts of the LHA apart from the CART-containing MCH neurons[13]. In regard to the extensive hypothalamic colocalization of CART-IR with both anorexigenic and orexigenic neurotransmitters, namely POMC at the Arc and MCH at the LHA and ZI respectively, it has been hypothesized that CART co-stored within POMC-IR neurons, functions to counteract the effects of MCH in feeding stimulation, on the assumption that CART and MCH may be co-released[13,96]. This is supported by the observation of an elevated CART tone in the MCH-IR LHA neurons in *MCH* knockout mice, which exhibit anorexic tendencies marked by hypophagia and a leaner phenotype[13,96]. Interestingly, no colocalization was found between CART and another orexigenic peptide confined to neurons in the LHA, orexin B[13,97-99], which increases in transcription activity during fasting to elicit stimulatory effects on feeding[99].

Colocalization experiments exploring the resemblance of CART distribution in feeding-related neuronal groups of the human hypothalamus revealed that, while overlap is seen in certain areas, some striking differences in colocalization pattern also exist[39,72,92]. For example, the close overlap of CART and POMC expression in rodents is not so obvious in the human brain[100,101]. In particular, CART expression could not be detected in the perikarya and axons of α-MSH-IR neurons but instead was found in approximately 30% of NPY/ agouti-related protein (AgRP) neurons[101] in the human infundibular nucleus analogous to the Arc in rodents[100]. However, similar co-expression patterns of CART and other neurotransmitters between human and rodents were seen in the PVN region[92]. Intriguingly, the co-expression of CART in NPY/AgRP instead of POMC neurons of the Arc was also observed in other primates such as monkeys[102]. Comparable with the observation in rodents, CART was demonstrated to colocalize with MCH in the lateral hypothalamus of humans, particularly in the perifornical region where up to 50% overlap in immunoreactivities of the two peptides was detected[92]. In the infundibular nucleus, fibres comprising MCH-IR were also observed in a portion of CART-IR axons, suggestive of lateral hypothalamic origin[92].

It is of particular interest that CART in humans has been shown to exhibit the contrasting colocalization with the orexigenic NPY/AgRP neurons and complete segregation from POMC-containing cells in the infundibular nucleus analogous to the rodent arcuate nucleus, whilst co-storage with MCH remained in the lateral hypothalamus. Speculations entailing an orexigenic role of CART as well as the involvement in other functions have therefore arisen, which is supported by the elevated food intake following CART administration to the PVN even in rodents[42]. Such paradoxical potentials of CART in feeding behaviors as evident in multiple pharmacological and physiological studies will be discussed subsequently in the present review. With regard to the resembling colocalization with the orexigenic MCH in the LHA consistent across species investigated[13,39,92,95], although CART expression appeared unaffected by alterations in energy homeostasis in the MCH neurons in rodents, MCH administration effectively blocked intracerebroventricular CART-induced stimulation of central dopaminergic neurons[103]. Indeed, lateral hypothalamic CART synthesis has been linked to the regulation of dopaminergic reward pathways[104], where psychostimulant-like effects were modulated by CART through moderating the activities of dopaminergic neurons[105-107]. Thus, while possibility exists that CART and MCH may participate in regulating different physiological functions effectuated by the same neuronal groups, anatomical and functional interplay of the two peptides has been indicated to prevail in the interactions between food intake and reward neurocircuitries[103,108,109]. Knowledge regarding the underlying cellular mechanisms awaits further extension through neuronal mapping and biochemical studies.

While the role of CART in controlling appetite and energy homeostasis in the human system might be somewhat different, in rodents, the neuronal network in which CART is involved to modulate energy

homeostasis has been well-described. The expression of endogenous CART at brain regions involved in feeding regulation has been shown to be sensitive to the energy balance status and the genetic background of mice. In brief, fasting has been documented in various mammals to reduce *CART* mRNA levels at the hypothalamic PVN, Arc, perifornical region, as well as the nucleus accumbens shell (AcbSh) of the striatum, whilst refeeding restored the expression[13,20,26,28,110-115]. As a leptin-regulated neurotransmitter, the expression of CART mRNA and peptide levels at the Arc is described to be positively correlated with circulating leptin levels[26,84,116-118]. This relationship between leptin and CART levels was less consistently demonstrated at the PVN and LHA[117], despite evidences supporting a pivotal role of CART-containing neurons projecting from the Arc to the second order neurons located in the PVN and LHA in producing anorexia[40,91,119]. Intravenous leptin administration was shown to induce Fos expression in hypothalamic CART neurons in the PVN, DMH, Arc, and the ventral premammillary nucleus[38,39]. Whilst Arc CART mRNA and peptide levels were strongly downregulated in food-deprived animals, the transcripts were nearly absent in genetically obese *fa/fa* rats and *ob/ob* mice with disrupted leptin signalling, wherein daily intraperitoneal leptin treatment led to *CART* restoration in the Arc and DMH[26,84,116,118,120], suggesting that leptin-induced anorexigenic actions may be mediated via CART neurons at the Arc. In comparison, in the *anx/anx* anorexia mouse model characterized by marked reduction in feeding and premature death, *CART* expression was significantly lower in the Arc, and less prominently in the DMH and LHA regions[121]. The reduced Arc *CART* expression, together with downregulated serum leptin levels, was attributed to a compensatory response to the energy-deprived state, as well as a probable molecular defect in the Arc deregulating the cellular source of *CART* mRNA[121]. In contrast with genetically obese animal models, diet-induced obese (DIO) rodents subjected to a high fat diet (HFD) have been shown to display higher Arc *CART* mRNA levels compared with lean animals fed a low fat diet, due to hyperleptinaemia[116,117,122]. Elevated *CART* transcript levels were also found in normal-weight obesity-prone rats compared to obesity-resistant subjects, where the Arc leptin-CART pathway was proposed to respond to fat-rich dietary intervention through inhibiting excessive body fat accrual by substituting lipid storage with lipid mobilization[116,117].

Despite purported relevance of CART in the modulation of gastrointestinal (GI) function, it is at present unclear the definite roles of enteric CART in intestinal motility, satiety and feeding behaviour[57,123-125]. Convincing evidences supporting the GI effects of CART have been provided by independent investigations, where central injections of CART in rodents elicited an anorexigenic response accompanied by the inhibition of gastric emptying and gastric acid secretion[124-126], while colonic motility measured by colonic transit time was accelerated[123]. Contrary to a local ENS effect, such alterations in GI functions were indicated to be conveyed through CART acting in the CNS[57,123-125,127], as intraperitoneal (i.p.) CART administration failed to reduce food consumption or reproduce similar gastric responses[123,124,128]. Furthermore, pretreatment with central injection of CRH receptor antagonist prior to central CART administration completely abolished the CART-induced gastric effects, suggesting a central CART-directed CNS modulation of the digestive tract behavioural motor functions via CRH-dependent mechanisms[123-125,127].

Consistent with a potential CART pathway connecting gut-brain signals in the control of food intake, co-expression of CART and the satiety factor cholescystokinin (CCK)-1 receptors has been detected in the *f* afferent neurons in rodents[22,61,62,129-134]. Substantially, the synergistic effect of CART and the anorexigenic gut hormone CCK[135] on food intake regulation analogous to that documented for leptin and CCK[61,136-138] has been demonstrated in animal studies, during which the responses to simultaneous as well as separate central administrations of CART peptide and the CCK octapeptide (CCK-8) through cannula implantation[139] were compared[140,141]. In fasted lean mice, the anorexigenic effect induced by CART delivery was significantly enhanced by parallel CCK-8 injection when compared with the administration of each particular peptide alone, while the additive reaction was also shown in an open field test where locomotor activity of the subjects was inhibited[140]. On the contrary, application of the CCK-1 receptor antagonist devazepide blocked CART-induced alteration

in food intake[140]. Such long-lasting cooperative action on feeding was speculated to associate in turn with the synergistic effect of leptin on CCK-induced satiety, where CART release from the nodose ganglia was mediated by the interaction of low-affinity vagal CCK-1 receptors and leptin receptors to produce short-term satiety[140,142]. Furthermore, 17% and 41% of vagal afferent neurons projecting from the nodose ganglia to the stomach and duodenum respectively were identified to be CART-immunoreactive[134]. The vagus has been proposed a mediator of the dorsal hindbrain action of CART on gastric motor function, as indicated by the inhibitory gastric effects of hindbrain intracerebroventricular (i.c.v.) CART microinfusion, which was blocked by subdiaphragmatic vagotomy[125,127]. In particular, the dorsal vagal complex (DVC) was demonstrated to be a target site for both CART and CRH in the suppression of gastric emptying, where effective inhibition occurred in response to the intraparenchymal injection of either peptide into the DVC, at a lower dose compared to that required to elicit a noticeable inhibitory effect by hindbrain i.c.v. administration[127].

Reciprocal of a central CART-directed modulation of gastric behaviour in feeding regulation, the anorectic actions of central CART have also been proposed to result from stimulation by gut hormones[143]. Following food intake, CCK released in the GI tract has been delineated to direct CART release from central vagal afferents, where an abundance of *CCK-1* receptor mRNA expression was detected in the nodose ganglion[61,143]. CCK-borne information is then mediated by CART to the hindbrain sites where suppression of food intake is elicited[61,143]. However, the role of CART released from vagal afferent terminals in the commissural part of the nucleus of the solitary tract (NTS) was suggested to be minor in mediating vagal satiety signals, as a diminished suppression of food intake was exerted by direct NTS subnuclei CART injections compared to hindbrain i.c.v. CART[128,134,144]. Thus, the potential function of CART on satiation may involve a distal site of action as well as interaction with other nutritional transmitters critical for vagally-mediated gastrointestinal satiety[61,134]. For instance, induction of CART immunoreactivity was readily demonstrated upon CCK administration both in rats and cultured vagal afferent neurons in a state of energy restriction, while refeeding of starved animals markedly increased CART immunoreactivity via a CCK-1 receptor antagonist-sensitive mechanism[62,145]. In addition, introduction of the orexigenic peptide ghrelin inhibited CCK-mediated CART stimulation[62], illustrating an interplay between gastrointestinal orexigenic and anorexic peptides in modulating CART expression. In particular, immunoreactive CART neurons have been shown to display differential quantities during developmental changes in the dorsal motor nucleus of the vagus (DMNV), wherein vagal preganglionic neurons are responsible for regulating various ingestive behaviors through innervations in the GI tract[133,134]. Such descending expression levels upon maturation indicate a potential signalling role of CART critical in the early postnatal development[133].

4. Peripheral CART in the regulation of energy homeostasis

The proposed physiological role of CART as an endogenous anorexigenic factor was originally deduced from the inhibition of food consumption observed in animal models following hypothalamic or intracerebroventricular administrations of CART-derived peptides[26,42,146-152]. Based on the dual involvement in both hypothalamic modulation of feeding behaviour and autonomic control of gastrointestinal functions common to many neuropeptides, considerable efforts have subsequently been devoted to the investigation of CART-mediated effects in the enteric nervous system (ENS)[11,57,123-125,153-155]. Extensive CART expression has been characterized in the ENS of diverse mammals by *in situ* hybridization[57] and immunoassays[11,153-156], affirming the presence of CART mRNA and peptides in nerve cell bodies and fibres innervating the stomach, small and large intestines of the gastrointestinal tract[58], particularly within the neuroendocrine cells and myenteric plexus[156]. Such brain-gut CART expression suggests that CNS control of feeding and satiety may be coordinated with local gastric CART-induced effects to produce an integrated regulation of body weight. This is supported by the central CART immunoreactivity profile, which depicts concentrated expression at

the hypothalamic nuclei[3,4,12,17,23-25,54-56] constituting major relays linking the sensory, motor and limbic areas between forebrain and hindbrain through widespread reciprocal networks, orchestrating autonomic, endocrine and behavioural activities[90,123,157,158]. Specifically, anatomical implications of CART in digestive function have been further indicated by CART immunoreactivity at the hypothalamic Arc and PVN neurons, as well as brainstem nuclei such as NTS and parabrachial nucleus (PBN), both involved in the efferent and afferent control of GI function through neuropeptidergic mechanisms engaging the complex neuroendocrine and autonomic pathways[26,42,90,123,144,147,159]. In addition, CART expression in the cholinergic neurons of the myenteric plexus and the pancreatic islets further denoted the potential gastric effects of CART conducted via peripheral receptor targets composing the peripheral cholinergic pathways[57,60,65,123,153].

The precise functions of CART peptides released by enteric CART-expressing neurons in the ENS are yet to be determined[58]. There has been a lack of direct evidence regarding a role of locally produced CART in classical neurotransmission within the GI tract, where intestinal motility as measured by contractile or relaxatory responses was unaffected by CART peptide application when motor activity studies were performed *in vitro* on muscle strips from stomach, small and large intestine[57]. Notably, exceptions to the above may include specific CART-evoked colonic responses, such as the attenuation of nitric oxide-induced intestinal relaxation[57,58], as well as the apparent stimulation of colonic transit, an indirect measure of colonic motor function[123]. In spite of the confined documentation of CART involvement in brain-gut interaction and the indeterminate functional role of enteric CART, accumulating evidences propose a role of CART in intestinal adaptation, where the survival and maintenance of enteric neurons is promoted[57,58]. Such neuroprotective property[58,160] and intestinal plasticity has been inferred from upregulated CART expressions and increased CART-expressing neurons in atrophic intestine and cultured myenteric neurons respectively, conditions indicative of neuronal stress or injury[57,58]. In summary, gastric involvement of CART has been evident through discrete histological and physiological experiments, whilst further detailed characterization of distribution and functions may contribute to a comprehensive understanding of the specific roles of enteric CART.

5. Functional implications of CART on energy metabolism from pharmacological interventions

The identification of the underlying mechanisms by which CART exerts effects on feeding and energy homeostasis have been challenging due to the lack of any knowledge of the corresponding CART receptor(s) and the absence of specific antagonists. Nevertheless, numerous studies incorporating both pharmacological and genetic manipulations of CART expression in murine models have been endeavored in the last decades to determine the sites of action and the effects on feeding behavior and metabolism of the peptide. Overexpression studies to discern the brain regions mediating CART-induced regulations of feeding have been the most common approach in rodents. Widely adopted as an appetite-regulating peptide of the CNS with hypothalamic expression levels modulated by nutritional status[8,18,26,36], CART was administrated i.c.v. to address the effects of overexpression during varying energy states[18,26,110,146-149,151,152,161-163] (Table 1). The lateral ventricle (LV) of the forebrain or the 3rd ventricle (3V) have been the major injection targets. I.c.v. injection of recombinant CART peptide has been consistently demonstrated to inhibit food intake and body weight gain in a dose-dependent manner in both food-restricted and free-feeding conditions as well as both under standard chow or a nutritionally complete liquid diet, in either normal or diet-induced obese animals[18,26,110,116,117,146-149,151,152,161-166]. Furthermore, the catabolic capacity of CART appeared sufficient to prevent and attenuate the orexigenic effects of NPY, as i.c.v. and intra-PVN CART potently suppressed feeding in satiated rats subjected to NPY-induced hyperphagia[26,40,110,116,148]. Similarly, the anorectic potency of CART has also been demonstrated in a recent study focusing on the interaction between CART and the GABA type A receptor (GABA-A) active neurosteroid allopregnanolone (ALLO) and the inhibitor neurosteroid dehydroepiandrosterone sulfate (DHEAs)[152].

It was shown in rodents that pre-treatment of i.c.v. CART effectively attenuated subcutaneous ALLO-induced hyperphagia and weight gain, as well as potentiating DHEAS-induced hypophagic and weight reducing effects[152].

CART administration via the i.c.v. route was also able to eliminate the increase in feeding and deleterious weight gain caused by social isolation in rats[151], a consequence of the downregulation of the hypothalamic CART-containing system in various hypothalamic feeding-related areas caused by this condition[151]. In the same study, whilst re-socialization of the isolation-reared rats restored the food intake, body weight, and hypothalamic CART-immunoreactivity back to controls levels, immunoneutralization of endogenous CART by i.c.v. CART antibody attenuated the restoration, confirming the important role of CART in feeding regulation under chronic psychological stress condition[151]. This is consistent with other studies that used antibodies raised against different CART segments for blocking central CART signaling, where all of which were able to neutralize the anorectic property of CART and lead to a significant hyperphagic response[26,110,151,167]. In addition to eliciting an anorectic response, gastrointestinal effects including inhibition of gastric acid secretion and gastric emptying have also been reported as a result from i.c.v. CART[123-127]. Chronic overproduction of *CART* mRNA via viral approaches or continuous infusion of recombinant CART peptide transferred through i.c.v. cannulas into genetically (*fa*/*fa*)[149] or diet-induced[116,165] obese rats induces hypophagic effects during fed states and reduced hyperphagia following fasting were also observed. Such reduction in energy intake was accompanied by suppression of body weight gain mainly due to decrease in lean mass[149,165], indicating the potential of CART in the long-term regulation of food consumption and body mass, under both normal condition and nutritionally induced obesity.

In conjunction with the characterization of physiological responses, neuronal activities stimulated by central CART has been investigated by structural studies for the purpose of identifying brain areas potentially crucial for CART-induced anorectic effects. Following i.c.v. CART administration, temporal expression patterns of the immediate early gene *c-Fos*[168,169], which has been adopted to depict neuronal firing of actions potentials[168,169], were found to concentrate in the hypothalamic and brainstem structures implicated in the central regulation of feeding[134,148]. In the hypothalamus in particular, high density of Fos expression was located in the PVN and the posterior DMH, while considerable Fos-IR cells were also identified in the Arc and SO. In the brainstem, Fos-positive cell nuclei were also concentrated in the PBN and, more importantly, in the NTS, which serves a key sensory relay nucleus with reciprocal connections with numerous forebrain and brainstem structures[148]. Such CART-induced Fos activation in the NTS has been indicated independent from possible secondary effects triggered by chemo-activation at the area postrema (AP) directed to the NTS, as the chemosensitive neurons in the AP were devoid of Fos-IR cells[148]. Moderately high Fos expression was also detected in cerebral nuclei associated with autonomic functions and energy balance[148,170], including the central nucleus of the amygdala, where neuronal projections also reciprocally link with the PVN of the hypothalamus and the PBN and NTS of the hindbrain[171,172]. The widespread Fos expression pattern elicited by forebrain i.c.v. CART has been demonstrated to encompass an anatomical continuum of neuronal activations across the cerebrum, hypothalamus and brainstem[148]. The paralleled effects on appetite inhibition and metabolic regulations are believed to portray an integrated outcome of the interactions between central CART-interfered pathways residing primarily within the hypothalamic and brainstem neurons. For instance, as aforementioned, the administration of CART combined with other neuromodulatory such as CCK in mice generated synergistic effects on food intake and locomotion, while displaying concomitant enhancement in the number of Fos-positive neurons compared to injecting each peptide alone[140,173]. The additive effect on Fos immunoreactivity was especially notable in the target areas common to both peptides, namely the hypothalamic PVN, DMH, VMH and Arc, as well as NTS at the brainstem[140,173], wherein the CCK-related satiety signals transmitted to the hindbrain were suggested to be further regulated by leptin action integrated in the Arc as well as neuronal signals from both PVN and LHA[136,140,174].

CART is widely expressed in the brain and particularly concentrated in the hypothalamus, suggestive of a diverse range of functions. While effective, delivery of ligands via the i.c.v. route is associated with the downside of the simultaneous stimulation of pathways in various parts of the brain, likely contrasting with effects attributable to the activation of specific neuronal populations. One such case are the CART neurons at the Arc, which respond to and are modulated by leptin signals, leading to the activation of selective neurons and associated downstream pathways[35,36]. It is therefore unsurprising that the observed effects of the i.c.v. injection of substances like CART are not always replicated by targeted delivery of the same peptide into specific nuclei of the hypothalamus. Indeed, several studies have shown that targeting CART into individual hypothalamic nuclei results in revelation of the orexigenic effects of CART, leading to increased food intake and body weight[41,122,164,175]. The strongest orexigenic effects were observed by injection of CART into the VMH, DMH and Arc, and a much lesser effect was observed when administered into the PVN, LHA, anterior hypothalamic area, and SO[41,122,164,175] (Table 1). Other effects following Arc and PVN CART delivery such as greater energy expenditure and thermogenic capacity, as indirectly measured by the expression and activity of UCP-1 in brown adipose tissue crucial in thermogenesis, has also been reported[40,41] (Table 1).

Despite the dependence of endogenous CART expression on nutritional states discussed above, energy states of the animals or dietary options appeared to have little influence over the potency of CART administration-induced feeding stimulation[41,122,164,175]. For instance, intra-arcuate delivery of CART resulted in elevated food intake in rodents under both fasted, food-restricted and satiated conditions, subjected to the dietary interventions of either regular chow or HFD[41,122,164]. Intriguingly, the orexigenic effects of CART were exhibited in both non-diabetic normal rats as well as streptozotocin-induced diabetic rats, where the intra-Arc CART-induced increase in feeding was reproduced under various energy states and dietary treatments[122]. Similarly, in rats receiving chronic overexpression of recombinant CART virally delivered into the PVN, higher cumulative food intake and body weight gain was observed in both groups fed either normal chow or HFD compared to control groups, with more pronounced changes in the HFD group[175]. Underlying such observations, the appetite-promoting effects of hypothalamic intranuclear CART administration may be attributed to a role of hypothalamic CART in stimulating the release of orexigenic neuropeptides locally[122,175]. This is supported by experiments involving a static incubation system where an increase in the release of NPY- and AgRP-IR but not α-MSH-IR was detected in both Arc-containing hypothalamic explants incubated with CART peptide *in vitro* as well as in PVN-containing hypothalamic explants isolated from animals subjected to intra-PVN CART injection[122,175]. Direct hypothalamic intranuclear CART injection at specific sites, therefore resulted in feeding behaviors opposite to the anorectic effects seen for i.c.v. CART.

The discrepancy between the anorexic effects of CART when injected i.c.v. versus the predominately orexigenic effects of CART when delivered into specific hypothalamic nuclei suggests that CART expression/function in other brain areas may also be important to the regulation of food intake and energy homeostasis, also suggesting that CART may be involved in both anorexigenic and orexigenic circuits in the CNS[176]. Other potential areas for CART-mediated anorexic effects include the striatum, which is known to have upregulated *CART* expression following acute i.p. administration of psychostimulants[3,23,177], reduced *CART* mRNA levels following fasting[20,26,28], and has been shown to be involved in the mediation of reward and reinforcement[178-180] as well as the neuronal circuits controlling feeding behavior[28,180-186]. Evidence for such a role was gained from experiments in a strain of CCK-1 receptor-deficient obese rats, where a significant reduction in CART immunoreactivity in the Arc was found potentially associated with a diminished anorectic effect of CART peptide compared to lean controls[187]. Furthermore, intra-accumbal CART peptide injection has been demonstrated to diminish both basal food consumption and food deprivation-induced feeding[28,29], as well as potently attenuating the orexigenic effects of the GABA-A agonist muscimol[28], albeit some inconsistency across different studies[188] (Table 1). The antagonistic effects of the GABA

system and CART at the Acb were also demonstrated in the neurochemical phenotypes of hypothalamic neurons after the appetite-inducing microinjection of muscimol into the AcbSh, which increased Fos expression in orexin neurons at the perifornical area and NPY neurons at the Arc, while inhibiting that in Arc CART/POMC neurons[189]. In a recent study, subcutaneous injection of the GABA-A active neurosteroid ALLO significantly reduced CART immunoreactive cells and fibers in the AcbSh, as well as in other feeding-related hypothalamic nuclei such as the PVN, Arc and LHA[152]. Direct CART administration into the Acb performed by an independent group generated no detectable influence on food reward assessed by food self-administration, yet triggered inhibitory effects on cocaine self-administration, an alternative measure of reward and reinforcement entailing dopaminergic functions[188].

The anorexia elicited by intra-accumbal CART was more sustainable in freely fed compared to starved animals, highlighting the significance of fuel status on CART function in feeding modulation[28]. Furthermore, complementary to the overexpression experiments, RNA-interference has been employed to investigate the effects of CART depletion in rodents[29,190]. Tissue-specific CART knockdown in the Acb via intra-accumbal administration of short interfering RNA (siRNA) or short hairpin (shRNA) against *CART* mRNA induced body weight gain and hyperphagia in fed mice[29,190], as well as abolishing the anorectic effects of serotonin (5-hydroxytryptamine, 5-HT) 4 receptor (5-HT4R) stimulation as well as 3,4-*N*-methylenedioxymethamphetamine (MDMA, ecstasy) treatment in starved mice, further denoting the potential role of Acb CART in mediating the appetite suppressant properties in models of anorexia nervosa[29].

Despite the body of evidence endorsing the plausibility of the Acb as a site for CART-directed anorexia, the appetite-regulating effects produced by intra-accumbal CART likely represent part of the reward and motivational responses derived from an interaction between CART and the dopaminergic system in the Acb. Multiple lines of evidence have suggested an inhibitory role of endogenous accumbal CART in addiction-relevant behaviors, which are speculated to act in concert with feeding modulation as well as the locomotive effects mediated by the dopaminergic circuits[180,188,190-192]. For instance, substantial innervations have been described for CART-containing neurons in the ventral pallidum, a key nucleus harboring accumbal efferents, where CART-IR terminals were reported to compose symmetric synapses resembling inhibitory GABAergic synapses[191]. Whilst intra-accumbal administration of CART alone produced no effect on locomotor activity, co-injection with cocaine or amphetamine into the Acb inhibited the cocaine-like locomotor effects produced by Acb dopamine microinfusions, both intra-accumbal and intra-pallidal injections of CART peptide led to reduction in cocaine- and amphetamine-induced locomotor activity[107,188,191-193]. Correspondingly, CART depletion through intra-accumbal CART shRNA increased cocaine-mediated locomotion[190], supporting an antagonistic property of Acb CART in the functions of cocaine and other psychostimulants.

The hindbrain, as a region described to convey post-prandial satiety effects to the hypothalamus, has been considered a potential candidate site for CART action[2,194]. Supporting evidences include the moderate CART expression in terms of both transcript levels and immunoreactivity in caudal brain areas such as the locus coeruleus, NTS, PBN and the inferior olive[3,22], accorded with the increased Fos-IR identified in the NTS and PBN following i.c.v. CART into the LV[148]. Comparable to i.c.v. injections into the LV or 3V, hindbrain delivery of CART peptide through the 4th ventricle (4V) led to reduction in food intake and body weight in both fed and food-deprived rodents, whilst the hypophagic effects showed no specificity to nutrients from either chow, sucrose or a nutritionally complete liquid diet[128,134,144,147,195] (Table 1). Importantly, the extent of feeding inhibition appeared more potent with CART administered into the 4V compared with LV injections[144], raising the speculation that the hindbrain may house the key mediator for the hypophagic effects of i.c.v. CART[147]. Foundation for the idea involved the postulate that the anorectic effects triggered by forebrain i.c.v. CART indeed reflected the outcome of CART diffusion into hindbrain sites via the cerebrospinal fluid (CSF)[147]. Surmised from the CART-IR observed in cell bodies and central

terminals of vagal afferent neurons projecting to the GI tract, a potential functional role of CART in meal termination and satiety may effectuate at the level of the brainstem[61,134]. In rat, vagotomy caused considerable reduction in *CART* mRNA expression in several CART fibers in the vagus nerve and viscero-sensory nodose ganglion[61]. To verify such proposition, cerebral aqueduct occlusion was performed with an aqueductal plug to interrupt the forebrain-hindbrain CSF flow, and CART was injected into the 3V or 4V[147] (Table 1). Interestingly, cerebral aqueduct blockage markedly attenuated the anorectic effects of 3V CART, whilst suppression of food intake remained unchanged when receiving 4V CART injection, signifying the independence of hindbrain CART in producing anorexia[147]. In contrast, hindbrain processing may be required or responsible for mediating a hypophagic response following forebrain or interbrain i.c.v. CART, further reinforcing the role of the brainstem in manifesting CART-driven anorectic effects, as concordant with the aforementioned higher potency in feeding inhibition with 4V as opposed to forebrain or interbrain i.c.v. CART[144]. Specifically, on the assumption that the repressed ingestive behaviors following LV or 3V CART may attribute to hindbrain CART action, the observations resulted from obtrusion of CSF flow could offer a possible explanation for the opposite feeding effects of orexigenic and anorexigenic natures induced by direct hypothalamic subnuclei[40,41,122,164,175] versus hindbrain ventricular[128,134,144,147,195] CART administrations respectively. Such phenomena promote reevaluation of the authenticity and proposed mechanisms involved in the hypophagia exhibited after forebrain ventricular CART detailed in other studies[26,110,146-149,164].

6. Functional implications of CART deletion on energy metabolism from genetic interventions

In order to gain further insight into the functional consequences of reduced or absent CART expression, several knockout models have been generated and characterized[66,196-201] (Table 2). The phenotypic effects resulting from *CART* gene targeting approaches have in part shown inconsistency between studies, however, displayed a general trend in promoting positive energy balance. This in overall terms is also similar with results obtained from our novel *CART* knockout (KO) model, which was generated from a conditional version that was crossed with an oozyte-specific Cre line. Collective evidence regarding the role of CART in energy homeostasis from a few representative studies conducted by independent research groups (Table 2) as well as in-house results will be discussed.

In all models investigated, the expected increase in feeding due to depletion of the anorectic CART peptide was not observed under standard chow feeding conditions. However, when exposed to a high caloric diet, lack of CART led to altered feeding behavior and body weight[66,196,197,200]. An increase in body weight has also been shown in *CART* KO mice fed on regular chow, although manifestation of such trait appears to require a longer time frame, where in one study, a statistically significant increase was absent at 17 weeks of age[196], whilst an independent group adopting the identical mouse model reported notably higher body weight for the knockout mice at 20 weeks[197]. In a different *CART* knockout mouse line, again altered weight was undetectable prior to 40 weeks of age[66,200]. Similarly, when fed a high fat diet, a significantly higher food consumption was observed in the *CART* KO compared to wild-type (WT) mice[196], in some but not all studies addressed. Importantly, despite a lack of consistent alteration in food intake, all studies reported an elevated body weight gain in *CART* knockout animals regardless of dietary options, with an even more prominent effect shown with a high caloric diet[66,196,197].

Interestingly, fasting-induced food intake experiments in our novel *CART* KO model suggest that lack of CART may be beneficial for body weight conservation during starvation. In our study, chow-fed *CART* KO animals showed a slightly lower food consumption compared with WT during a refeeding period following a 24-hour fast, yet demonstrated a similar degree and pace of recovery in body weight, implying that less food might be required for returning to the pre-fast weight. On the other hand, the same *CART* KO mice on HFD experienced a less dramatic drop in body weight upon

fasting, and accordingly showed a more effective weight regain to baseline during refeeding. Such improved reactions to food deprivation may be correlated to the observed difference in growth and possibly attributable to events in the interaction between CART and an improved stress response in *CART* KO mice, consistent with a role of the CART system in stress and anxiety-like responses [1,41,42,59,151,202-208]. For example, i.c.v. CART has been reported to substantially influence the plasma levels of various stress hormones, such as adrenocorticotropic hormone and corticosterone[42]. Furthermore, anatomical implication has also been provided by the expression of CART transcript and peptide at various levels of the HPA axis as well as other stress-related areas in the CNS[1,3,12,22,24,38,54,59,151,209].

Amongst a multitude of potential factors contributing to the enhanced body weight in *CART* KO models, the gain in fat mass has been considered the most important[196]. This is concordant with results from our novel *CART* KO model, where body composition analysis by dual-energy X-ray absorptiometry (DXA) revealed a pronounced increase in adiposity independent of diet. The gain in whole body fat mass was further confirmed by tissue dissection showing significantly elevated fat masses in all white adipose tissues, including the inguinal, epididymal, mesenteric and retroperitoneal regions. This also suggests an important function of CART in lipid metabolism, where CART has been linked to inhibition of lipogenesis and stimulation of lipid substrate mobilization and utilization[69,70,116,117].

In addition to fat mass, lean mass constitutes another major determinant of energy homeostasis that directly influences energy expenditure. As opposed to the increase in fat mass, whole body lean mass of our CART-deficient mice was distinctly lower compared to WT controls regardless of dietary treatments. Similarly, the reduction was consistent across both periods of DXA measurements at 10 and 14 weeks of age. Intriguingly, research investigating the effects of chronic central CART infusion in genetically normal DIO rats reported a diminished body weight gain primarily due to a loss of lean mass[149,165], while fat mass was unaffected[165].

Consistency exists between results from our *CART* KO model and previous studies focusing on the metabolic characterization of *CART* knockout mice, where no significant difference in total energy expenditure or physical activity could be detected when compared with WT controls regardless of diets[196,197], even after the correction of the potentially confounding effects of lean mass on energy expenditure. However, overexpression studies have provided indications of both supporting and opposing roles of CART in regulating energy expenditure[41,165]. An increase in energy expenditure was suggested in rats subject to chronic overexpression of CART through intra-arcuate targeted gene transfer, where the animals showed exaggerated weight loss and a downregulation of endogenous arcuate *CART* mRNA levels upon fasting and food restriction[41]. Despite discordance between various animal studies, evidence exists for the involvement of CART in the regulation of energy expenditure and body weight in humans[43-49]. Specifically, a missense mutation in the pro-CART transcripts was discovered to co-segregate with a severe obese phenotype and was also associated with decreased resting energy expenditure in members of an Italian family over three generations[43,46,47].

In spite of a lack of direct indication endorsing a function of CART in modulating energy expenditure, comparing the respiratory exchange ratio (RER) between *CART* knockout animals and WT counterparts may shed light on any potential effects of CART ablation on energy metabolism through the fuel source preferences. Although no measurable difference was described in knockout animals in the respiratory quotient derived from RER as reported by previous studies[196,197], a notably lower RER has been detected in our new *CART* knockout mice. Consistency in the lower RER particularly during the dark photoperiod was shown across both nutritional statuses, signifying that fat was preferentially metabolized over carbohydrates to supply energy for the body. A possible explanation for this could be the higher fat content in knockout animals, which may lead to the predominant fuel source based on the relative higher availability of fat than carbohydrates. On the other hand, a suppressed average

respiratory quotient was demonstrated in both normal and DIO rats chronically overexpressing central CART compared to vehicle-treated controls[116,165]. The reduction was exaggerated during the dark phase, under both regular feeding and fasting-refeeding conditions, indicating a stimulatory role of CART in promoting lipid oxidation and limiting fat storage, hence inhibiting excessive body fat accrual[116,117,165].

Taken together, results from the literature as well as in-house studies of *CART* knockout models generally support the property of CART as a satiety factor and an anorexigenic signal in the brain, as evident in the elevation in body weight gain attributable mostly to the increased fat mass consistent across studies, although controversy exists for the corresponding food intake data. As for the aspect of energy intake, RER was demonstrated to be reduced both under the conditions of CART overexpression and ablation, suggesting fat was metabolized as the primary fuel for energy supply. A possible reason could be that although CART may intrinsically promote the utilization of fat as the predominant fuel source for reducing energy intake, the effectiveness of CART deletion on the disturbance of lipid metabolism hence accumulation of adiposity may have surmounted the simultaneous CART deficiency-induced enhancement of energy intake, resulting in a net reduction in RER based on the readily available fat depots.

7. Roles of CART in human

As introduced earlier, evolutionary conservation has been demonstrated for CART in the neuroendocrine system across various mammalian species in the contexts of isoform structure, expression distribution pattern as well as functional implications, including a role of CART in the regulation of energy balance in human[43-50]. First, a genome-wide scan for human obesity-susceptibility loci in obese French Caucasian families[50] revealed a clear linkage to the chromosomal locus of 5q13.2 where the human *CART* gene is encoded. Respectively, the expressions of CART transcripts and peptides have been characterized in various hypothalamic areas involved in appetite control[39,72,92], as well as in the subcutaneous and visceral white adipose tissues central to the moderation of lipid homeostasis[69,70]. Intriguingly, the aforementioned anatomical-functional implications provided by the expression patterns of CART in the human infundibular nucleus, which demonstrated colocalization with the orexigenic NPY/AgRP and segregation from the anorexigenic POMC neurons, had rendered a primary anorectic role of CART appealable[92].

In human, alterations in *CART* have been associated with reduced metabolic rate, hyperphagia, obesity and elevated incidence of type II diabetes[69]. For example, a Leu34Phe missense mutation in the human pro-CART transcripts was discovered in obese members of an Italian family across three generations to affect post-translational processing, which coincided with CART peptide deficiency in the sera and reduced resting energy expenditure, ultimately leading to hyperphagia and severe early-onset obesity[43,46,47]. In brief, the mutation was identified to neighbor a cluster of basic amino acids, hence presupposed to influence the specific processing of the pro-CART (1-89) in generating the biologically active CART I (42-89) and CART II (49-89) peptides[8,11,43,46]. To simulate cellular effects of the mutation, subsequent investigations transfected human *CART* cDNA constructs representing either the wild-type or the mutant into corticotropic AtT-20 cells, a mouse pituitary-derived cell line often used for studying peptide processing and, more importantly, is known to express and process CART peptides[46]. Notably, in addition to reduced CART peptide levels in cells transfected with the mutated cDNA compared with controls, expression of the mutated pro-CART was also described to be mis-sorted, poorly processed and secreted, thus disarranging the cellular distribution of CART as a whole[46]. Other than discoveries that addressed the potentially crucial role of protein biosynthesis in the development of obesity, the majority of the studies directed at the association between CART and obesity focused on polymorphisms in *CART* within populations.

Polymorphism studies in the *CART* gene conducted worldwide have established substantial linkage between a few specific single nucleotide polymorphisms (SNPs) to obesity[45,48,49]. For instance, a family-based association study of 133 Italian trios has identified significantly higher allele frequencies of the 1475A>G SNP in the *CART* gene in overweight and obese children compared to non-obese unrelated controls consisting of both adults and children, while preferential transmission of the allele to overweight or obese children from heterozygous parents was predicted[48]. In another Caucasian population, 292 French morbidly obese subjects were recruited for sequence variability screen in the *CART* gene, where three SNPs residing in the promoter region, with SNP-3608T>C in particular, were suggested by haplotype analysis to prominently contribute to the genetic risk for obesity[49]. The proposed association was further strengthened by the high prevalence of the specific allele in an expanded genotyping study, with additional populations of European Caucasian origin comprising 619 moderately obese French subjects and 385 morbidly obese Swiss subjects[49]. Extended on the genetic studies, plausible functional effects of the SNP were also investigated by electrophoretic mobility shift assays in cellular system, where modulation of nuclear protein binding affinity was demonstrated to potentially correlate with the obesity phenotype[49]. Besides Caucasians, a sequencing study in 528 Japanese subjects revealed a high level of polymorphisms in the 5'-flanking region of the *CART* gene housing the putative promoter region, wherein specific polymorphic sites or variants in linkage disequilibrium with the respective sites were identified to associate with genetic predisposition to obesity[45].

As mentioned, CART has recently been defined as a component of adipocytes involved in lipid substrate utilization in both human and rodents[69]. Investigation in a large Caucasian population of approximately one thousand subjects in the United Kingdom identified two common polymorphisms in the 3'-untranslated region of *CART*, that were implicated to interfere with fat distribution and contribute to dyslipidaemia[1,44]. Despite a lack of correlation with obesity through systematic mutational analysis, a particular genetic variant 1475A>G among the polymorphisms was illustrated to significantly affect waist-to-hip ratio as well as the levels of plasma insulin and triglycerides[44], suggesting a putative pivotal role of CART in glucose- and lipid-homeostasis. Consolidation of the proposition has been illustrated in subsequent haplotypic study in a general population of 840 subjects from North of France[70], a continuum from the former French project on *CART* promoter SNPs[49], where the three previously identified SNPs were described to affect plasma lipoprotein-cholesterol level and consequently associated with cholesterol metabolism and atherogenicity[49]. Specifically, the functional SNP-3808C>T was of particular interest, as plasma lipid profile traits protective against atherogenesis were displayed in cases bearing the allele, exemplifying the clinical potentials of *CART* in lipid metabolism and atherogenesis[49].

Taken together, human studies based principally on genetic polymorphisms have provided evidences promoting a role of CART in body weight regulation in humans. Altered *CART* expression has generally been associated with an elevated genetic predisposition to overweight and obesity, indirectly substantiating the anorexigenic nature of the peptide, although results from the literature show both anorexigenic and orexigenic properties of CART in animal studies. It is also noteworthy to address the plausible challenges imposed on the translatability of results obtained from animal models to the human system, considering the discernible difference in the anatomy of central CART-containing neurons between the two, as discussed above. Furthermore, although overall support has been gained for the hypothesis that inherited variations in *CART* could influence the development of obesity, such genetic linkage was absent for some other sequence variants detected within the gene, where the polymorphisms have been speculated as insufficient to disturb the peptide structure or create topological and conformational changes in the protein that would ultimately affect the functional activity of the peptide[1,210,211]. Indeed, recent studies conducting an alanine scan for assessing the importance of the structure-activity relationship of CART demonstrated the dependence of anorexigenic potency on individual disulfide bridges in the peptide[139,212,213]. To elucidate the contribution of specific disulfide bridges to maintaining the stability and biological function of CART,

analogs with only one or two among the three disulfide bridges in the intact peptide were synthesized, with which binding activities as well as metabolic effects were measured in both cell and animal systems[213]. Intriguingly, results from binding experiments in PC12 rat pheochromocytoma cells[139,213,214] indicated that the preservation of two particular disulfide bridges as well as the full-length peptide was imperative for biological activity, where high affinity of the analog to PC12 cells in both states of native phenotype and differentiated into neurons was measured[212]. In mice subjected to i.c.v. administration of the same analog, strong and long-lasting anorexigenic potency was exhibited during food consumption and behavioral tests, further purporting that one particular disulfide bridge could be omitted without a loss of bioactive function[212].

In summary, the familial nature of obesity is well-established to be interrelated with a prominent genetic component. The CART system has been evident to constitute a dominant player in feeding control, body weight regulation and energy metabolism, hence a promising candidate for the development of anti-obesity therapeutics. Respectively, population genetics have revealed the potential contributions of polymorphisms in the *CART* gene to abnormalities in feeding and body weight control, where effects on interactions between the transcription factors and regulatory elements binding to the polymorphic sites may exert phenotypic influence. However, elucidating the mechanisms of CART action as well as investigating and replicating the fine genetic mapping in further populations will be essential for unraveling the authentic role of CART in energy homeostasis and understanding obesity.

7. Conclusion

In conclusion, the widely adopted role of CART in the regulation of energy homeostasis has been summarized in this review from the perspectives of genetic and transcriptional associations, anatomical-functional correlation, pharmacological and genetic intervention studies in animal models, as well as implications from sequence variability in human. Nevertheless, the lack of a known CART receptor(s) and specific antagonists, continues to constitute the major challenge in understanding the underlying mechanisms by which CART exerts effects on feeding and neuroendocrine regulation.

The physiological importance of CART is endorsed by the evolutionarily conserved expression patterns in the brain as well as the periphery at regions associated with energy balance, inferring structural hence functional conservation in glucose sensing, lipogenesis regulation and ultimately the control of feeding behaviour. The resultant integrated outcome on metabolic modulation also represents the neuronal crosstalk involving similar neuromodulatory peptides between central CART-interfered pathways residing at the hypothalamic and brainstem neurons as well as via the hypothalamic-pituitary-adrenal axis. In comparison with CART in the brain, functional roles of peripheral CART are less established, with indeterminate modes of action speculated to entail either a local independent response, or synergistic effects with central CART.

Overexpression of central CART through i.c.v. injections at either the forebrain (LV), interbrain (3V) or hindbrain (4V) areas has confirmed an anorexigenic role of CART, while the appetite-promoting effects of CART administered into specific neuronal targets may be attributed to a role of hypothalamic CART in stimulating the local release of orexigenic neuropeptides in a hypothalamic nucleus-specific manner. The discrepancy in anorexigenic and orexigenic circuitry highlights the major pitfall of non-specific widespread effects associated with i.c.v. ligand delivery, and was recognized through the efforts to identify potential sites for CART action following the analysis of Fos expression pattern representative of i.c.v. CART-induced neuronal activities. Candidate sites that may house the key mediator for the hypophagic effects of i.c.v. CART primarily reside in various feeding-related areas, including major hypothalamic nuclei, specific brainstem structures, and the Acb of the cerebral striatum. The appetite-inhibiting and -promoting effects produced by intra-accumbal

CART and *CART* siRNA respectively reflect a concerted response from the CART-dopaminergic system interaction within the Acb, hence the observed feeding modulation and locomotive effects were likely perplexed by the inherent reward and motivation pathway. Several hindbrain areas are of particular interest as alternative brain regions for CART-induced anorexic effects, owing to the well-described role of the hindbrain in conveying post-prandial satiety effects to the hypothalamus, consonant with the indicated relation between CART and vagally-mediated gastrointestinal satiety.

In parallel with overexpression approaches, independent research groups have adopted genetic ablation to generate several germline *CART* knockout murine models, to determine effects of CART depletion on feeding behavior and metabolism. General consistency has been demonstrated between results from the literature and in-house data from our novel *CART* KO model, supporting CART as a satiety factor. Collectively, the phenotypic effects of CART deficiency from birth, although inconsistent across studies, display promotion towards positive energy balance. Extended findings stemming from our novel KO model also denote that CART deficiency may confer an advantage in body weight conservation during starvation, potentially associated with an improved stress response attributable to an altered anxiety-related cascade involving the CART system.

Both gene knockout and overexpression studies of CART have consolidated the stimulatory role of CART in promoting lipid oxidation and inhibiting fat accrual. As discussed above, despite the widely documented anorectic evidence of CART, possibility of an orexigenic role is indisputable. Under certain circumstances, such postulation may provide explanation for the inconsistent or lack of marked phenotypes in terms of energy balance observed in germline *CART* knockout animals, as well as conditional, developmental stage, or whole brain *CART* knockout models. Nevertheless, temporal or tissue-specific genetic ablation serves an invaluable means to minimize common pitfalls associated with a germline knockout strategy, such as any secondary effects or compensatory mechanisms during development.

In human, the expression of CART transcripts and peptides has been characterized in various hypothalamic areas involved in appetite control and lipid homeostasis. From the perspective of population genetics, genome-wide study of human obesity-susceptibility loci revealed linkage to a locus harboring the human *CART* gene, whereas mutation profiling has associated alterations in *CART* with reduced metabolic rate and resting energy expenditure, hyperphagia, obesity and elevated incidence of type II diabetes. Intriguingly, whilst a role of CART in regulating energy expenditure remains equivocal in overexpression studies and considered discordant in gene deletion approach in animal models, mutation screening in humans has consolidated the involvement of CART in the modulation of energy expenditure, body weight and dyslipidaemia.

To elucidate the underlying cellular mechanisms, functional effects of mutations and polymorphisms that were linked to the development of obesity were investigated. The sequence alterations of interests were described to potentially interfere with the post-translational processing of CART, leading to defective protein biosynthesis and deranged cellular distribution of the peptide, while modulation in the nuclear protein binding affinity was also reported to attenuate biological activity. Despite the plausible challenges imposed on the translatability of principal observations from animal models to the less well-documented human system, aberrations in CART have been demonstrated in human studies to promote positive energy balance, endorsing a primary anorectic role of CART.

In summary, the CART system remains a dominant player in the regulation of feeding, body weight and energy metabolism, hence a promising candidate for the development of anti-obesity therapeutics. However, elucidating the underlying mechanisms of CART action, developing relevant pharmacological tools and understanding the nature of the endogenous CART receptor(s), remains crucial in unraveling the functional role of CART in energy homeostasis and obesity.

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Table 1. Summary of the metabolic and behavioral effects of central CART administration via various intracerebroventricular and intranuclear delivery methods. CSF, cerebrospinal fluid; DIO, diet-induced obese; HFD, high fat diet; BAT, brown adipose tissue; i.c.v., intracerebroventricular; i.c., intracisternal; LV, lateral ventricle; 3V, third ventricle; 4V, fourth ventricle; Acb, nucleus accumbens; AcbSh, nucleus accumbens shell; AHA, anterior hypothalamic area; Arc, arcuate nucleus; DMH, dorsomedial nucleus; LHA, lateral hypothalamic area; NTS, nucleus of the solitary tract; PBN, parabrachial nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus; VMH, ventromedial hypothalamic nucleus.

Year	Publication	Targeting peptide/ CART fragment	Route of adminis-tration	Species (genetic background)	Diet	Feeding behavior and body weight alterations	Locomotor behavior
1998	Kristensen *et al*. 1998 *Nature*[35]	CART I (55-102)	i.c.v.	Rat	Standard chow	↓ Spontaneous feeding; ↓ Fast-induced feeding; ↓ NPY-induced feeding	N/A
1998	Lambert *et al*. 1998 *Synapse*[215]	CART (55-59)	i.c.v.	Rat	Standard chow	~ Spontaneous feeding	N/A
1998	Lambert *et al*. 1998 *Synapse*[216]	CART (55-76)	i.c.v.	Rat	Standard chow	↓ Spontaneous feeding; ↓ NPY-induced feeding	N/A
1998	Lambert *et al*. 1998 *Synapse*[215]	CART (62-76)	i.c.v.	Rat	Standard chow	↓ Spontaneous feeding	N/A
1998	Thim *et al*. 1998 *FEBS Lett.*[18]	CART I (55-102); CART II (62-102)	i.c.v.	Mouse	Standard chow	↓ Fast-induced feeding	N/A
1999	Vrang *et al*. 1999 *Brain Res.*[148]	CART I (42-89)	i.c.v.[g]	Rat	Standard chow	↓ Spontaneous feeding (in food-restricted animals) & ↓ NPY-induced feeding	N/A
2000	Edwards *et al*. 2000 *Brain Res*.[161]	CART I (55-102)	i.c.v.	Rat	Standard chow	↓ Spontaneous feeding	N/A
2000	Kast *et al*. 2000 *Brain Res*.[162]	CART (62-76)	i.c.v.	Rat	Standard chow	↓ Spontaneous feeding	N/A
2000	Volkoff *et al*. 2000 *Brain Res*.[150]	CART I (55-102); CART (62-76)	i.c.v.	Goldfish	Standard chow	↓ Spontaneous feeding & ↓ NPY-induced feeding (in food-restricted animals)	N/A
2000	Larsen *et al*. 2000 *Obesity*[149]	CART I (42-89)	i.c.v. (chronic infusion)	Lean and obese Zucker (*fa/fa*) rats	Standard chow	↓ Spontaneous feeding (in both free-feeding & food-restricted animals); dose-dependent ↓ in body weight	Dose-dependent motor disturbances (combined gait and walking ataxia)
2000	Okumura *et al*. 2000 *Endocrinology*[217]	CART I (55-102)	i.c.	Rat	Standard chow	↓ Fast-induced feeding[b]	N/A

Year	Publication	Targeting peptide/ CART fragment	Route of adminis-tration	Species (genetic background)	Diet	Feeding behavior and body weight alterations	Locomotor behavior
2000	Wang *et al*. 2000 *Neuroreport*[40]	CART I (55-102)	intra-PVN	Rat	Standard chow	↓ NPY-induced feeding[i]	N/A
2001	Bannon *et al.* 2001 *J. Pharmacol. Exp. Ther.*[163]	CART I (55-102); CART II (62-102)	i.c.v.	Mouse	Standard chow	↓ Fast-induced feeding	N/A
2001	Abbott *et al*. 2001 *Endocrinology*[164]	CART I (55-102)	Hypothalamic intranuclear injections (VMH, Arc, PVN, SON, DMH, LHA & AHA)	Rat	Standard chow	↑ Spontaneous feeding (only measured in satiated animals cannulated into the DMH or Arc); ↑ Fast-induced feeding	N/A
2001	Abbott *et al*. 2001 *Endocrinology*[164]	CART I (55-102)	i.c.v. (3V)	Rat	Standard chow	↓ Spontaneous feeding; ↓ Fast-induced feeding (↓ feeding episodes)	Behavioral abnormalities marked by reduced feeding episodes, flat-backed posture & movement-associated tremors (behavioral analysis performed for 24-h fasted animals only but not satiated animals)
2001	Zheng *et al*. 2001 *Brain Res.*[144]	CART I (55-102)	i.c.v. (LV & 4V)	Rat	Sucrose solution or standard chow	↓ Spontaneous feeding (↓ short-term sucrose intake & ↓ overnight chow intake) - effects more pronounced in 4V compared to LV administration	Alterations in motor behavior (mild movement-associated tremors in part of 4V injected subjects)
2001a	Aja *et al* 2001a *Am. J. Physiol. Regul. Integr. Comp. Physiol.*[146]	CART I (55-102)	i.c.v.	Rat	Ensure liquid diet	↓ Spontaneous feeding (↓ liquid diet intake in licks and meal size in food restricted animals)	Altered oral motor function & behavioral alterations (trance-like state, flat-backed & arched-backed postures, cage licking, movement-associated tremors)
2001b	Aja *et al* 2001b *Am. J. Physiol. Regul. Integr. Comp. Physiol.*[147]	CART I (55-102)	i.c.v. (3V)	Rat	Ensure liquid diet	↓ Spontaneous feeding (↓ liquid diet intake and observations of feeding in food restricted animals) - reductions significantly attentuated by aqueduct obstruction[a]	Alterations in motor behavior (flat-backed & arched-backed postures & movement-associated tremors) - alterations significantly attentuated by aqueduct obstruction[a]
2001b	Aja *et al* 2001b *Am. J. Physiol. Regul. Integr. Comp. Physiol.* [147]	CART I (55-102)	i.c.v. (4V)	Rat	Ensure liquid diet	↓ Spontaneous feeding (↓ liquid diet intake and observations of feeding in food restricted animals) - reductions unaffected by aqueduct obstruction[a]	Alterations in motor behavior (flat-backed & arched-backed postures & movement-associated tremors) - alterations unaffected by aqueduct obstruction[a]

Year	Publication	Targeting peptide/ CART fragment	Route of adminis-tration	Species (genetic background)	Diet	Feeding behavior and body weight alterations	Locomotor behavior
2002	Aja *et al*. 2002 *Behav. Neruosci.*[195]	CART I (55-102)	i.c.v. (4V)	Rat	Ensure liquid diet	↓ Spontaneous feeding (↓ liquid diet & water intake in food restricted animals); production of conditioned taste aversion	N/A
2002	Rohner-Jeanrenaud *et al.* 2002 *Int. J. Obes. Relat. Metab. Disord.*[116]	CART I (55-102)	i.c.v. (chronic infusion)	Rat (normal and DIO)	Standard chow or HFD	↓ Spontaneous feeding & ↓ NPY-induced feeding; ↓ body weight gain	N/A
2002	Zheng *et al*. 2002 *Brain Res.*[218]	CART I (55-102)	i.c.v. (4V)[h] & intra-NTS	Rat	Sucrose solution or standard chow	↓ Spontaneous feeding (↓ short-term sucrose intake) - effects more pronounced in 4V compared to intra-NTS administration	N/A
2003	Smedh & Moran 2003 *Am. J. Physiol. Regul. Integr. Comp. Physiol.*[125]	CART I (55-102)	i.c.v. (4V)	Rat	Sucrose solution	↓ Spontaneous feeding (↓ sucrose intake in food restricted animals); altered lick microstrcuture parameters[c]	N/A
2003	Kong *et al.* 2003 *FASEB*[41]	CART I (55-102)	intra-Arc[d]	Rat	Standard chow	↑ Spontaneous feeding (in both free-feeding & food-restricted animals) & ↑ Fast-induced feeding; ↑ cumulative body weight gain; ↑ body weight loss following 24-hr fasting and food restriction[i]	N/A
2004	Wortley *et al*. 2004 *Regul. Pept.*[117]	CART I (55-102)	i.c.v. (3V)	Rat	Standard chow	↓ Spontaneous feeding	N/A
2005	Yang *et al*. 2005 *Neuroscience*[28]	CART I (55-102)	intra-AcbSh	Rat	Standard chow	↓ Spontaneous feeding; ↓ Fast-induced feeding; ↓ GABA-A agonist muscimol-induced feeding	N/A
2007	Qing & Chen 2007 *Regul. Pept.*[165]	rat CART cDNA	i.c.v.[e]	Rat (DIO)	High fat/high sucrose diet	↓ Spontaneous feeding; ↓ Fast-induced feeding; ↓ body weight gain (↓ lean mass; fat mass unaffected)	N/A
2007	Jean *et al*. 2007 *PNAS*[29]	CART I (55-102)	intra-AcbSh	Mouse	Standard chow	↓ Fast-induced feeding	N/A

Year	Publication	Targeting peptide/ CART fragment	Route of adminis-tration	Species (genetic background)	Diet	Feeding behavior and body weight alterations	Locomotor behavior
2007	Jean *et al*. 2007 *PNAS*[29]	CART siRNA	intra-AcbSh	Mouse	Standard chow	↑ Spontaneous feeding & ↓ stimulating 5-HT_4R- or MDMA-induced anorexia in staved animals	N/A
2008	Smith *et al*. 2008 *Obesity* (*Silver Spring*)[175]	rAAV encoding full length rat CART cDNA (GenBank accession no. U10071)	intra-PVN[e]	Rat	Standard chow or HFD	↑ Cumulative feeding & cumulative body weight gain; effects more accentuated on HFD	N/A
2009	Skibicka *et al*. 2009 *J. Neurosci.*[128]	CART I (55-102)	i.c.v. (4V) or intra-NTS	Rat	Standard chow	[4V injection][f] ↓ Spontaneous feeding & body weight (in food-restricted animals); hypophagic response and weight loss attenuated by pre-treatment with hindbrain delivery of GLP-1R antagonist (exendin-9-39); intra-NTS injection produced no observable effect on feeding or body weight	N/A
2010	Hou *et al*. 2010 *Clin. Exp. Pharmacol. Physiol.*[122]	CART I (55-102)	intra-Arc; intra-DMH	Streptozotocin-diabetic rats	Standard chow or HFD	Chow diet: ↑ Spontaneous feeding (in satiated animals)(Arc) & ↑ Fast-induced feeding (DMH & Arc); HFD: ↑ Spontaneous feeding (Arc)	N/A
2011	Nakhate *et al*. 2011 *Int.J. Obesity*[151]	CART I (54-102)	i.c.v.	Rat	Standard chow	↓ Spontaneous feeding & body weight; attenuated social isolation-induced hyperphagia & body weight gain	N/A
2013	Nakhate *et al*. 2013 *Brain Res.*[152]	CART I (54-102)	i.c.v.	Rat	Standard chow	↓ Spontaneous feeding; attenuated ALLO-induced hyperphagia & weight gain; potentiated DHEAS-induced anorexia & weight loss	N/A

Effects on body weight described where results presented, otherwise either unaffected or information unavailable.

Effects on locomotor behavior described where results presented, otherwise either unaffected or information unavailable.

[a] Cerebral aqueduct occlusion to interrupt forebrain-hindbrain CSF flow.

[b] Inhibition of gastric function (suppression of gastric acid secretion and gastric emptying); inhibition of gastric acid secretion remained in vagotomized animals; inhibition of gastric acid secretion blocked by pretreatment with central administration of CRF receptor antagonist α-helical CRF9-41.

[c] Inhibition of gastric function (suppression of gastric emptying); inhibition of gastric emptying blocked by pretreatment with central administration of CRF receptor antagonist α-helical CRF9-41; CART-induced inhibition of gastric emptying proposed unlikely to contribute to CART-mediated inhibition of food intake.

[d] Acute administration through repeated injections and chronic overexpression using stereotactically targeted gene transfer.

[e] Chronic overexpression using recombinant adeno-associated virus vector containing rat CART cDNA.

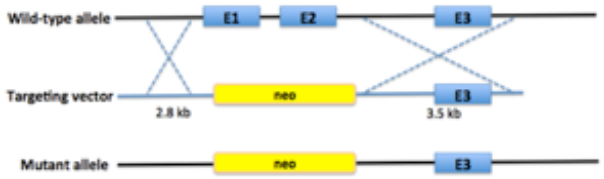
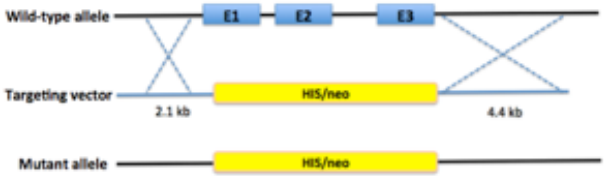
[f] ↑ Blood glucose levels; hyperglycemic response not altered by GLP-1R blockade in animals pre-treated with GLP-1R antagonist (exendin-9-39).

[g] Induction of Fos expression in the PVN, DMH, SON and Arc (hypothalamus), central nucleus of amygdala (cerebrum), PBN and NTS (hindbrain).

[h] Induction of Fos expression in NTS neurons.

[i] ↑ UCP-1 expression thermogenic capacity in BAT

Table 2. Summary of the metabolic phenotypes of germline *CART* knockout mouse models in various studies when compared with wild-type controls. Schematic representation of the gene targeting strategies adopted for generating *CART* knockout mouse models, depicting the wild-type allele of the mouse *CART* gene, the *CART* targeting vector, and the mutant *CART* allele. The three exons of the mouse *CART* gene are indicated by boxes labeled E1, E2 and E3. In the targeting constructs, the yellow boxes labeled "neo" or "HIS/neo" indicate the neomycin or histidinol/neomycin resistance selection cassettes respectively for targeted disruption of the *CART* gene. Homologous recombination between the targeting vectors and the complementary *CART* genomic loci (dotted lines) in mouse embryonic stem cells generates genetic ablations of exons E1 and E2, or all of exons E1, E2 and E3 of the *CART* coding region respectively. The targeting constructs were introduced into 129 SvJ mouse embryonic stem cells and subsequently injected into C57BL/6 blastocysts. M, male; F, female; HFD, high fat diet; NSD, no significant difference.

Publication	Targeting technique	Genetic Background	Diet	Feeding behavior	Body weight	Body composition	Energy and glucose homeostasis
Asnicar *et al*. 2001 *Endocrinology*	pGKneo gene cassette replacing CART exons I & II 	C57BL/6 mice	Normal chow	NSD	No change at 17 week	NSD	N/A
		C57BL/6 mice	HFD	↑ in *CART* -/- (M & F) & *CART* +/- (F only)	↑ in *CART* -/- (M & F) & *CART* +/- (F only)	↑ Fat mass in *CART* -/- (M & F) & *CART* +/- (F only)	↑ Serum leptin correlated with fat mass in *CART* -/- (M & F) & *CART* +/- (F only)
Wierup *et al*. 2005 *Regul. Pept.*	Cartpt^{tm1Amgn} replacing CART coding sequence exons (I-III) with HIS/Neo cassette 	Black swiss X 129SvJ (M only)	Normal chow	NSD	↑ at 40 week	N/A	Impaired glucose-stimulated insulin secretion both *in vivo* (glucose intolerence: ↑ basal insulin, normal baseline glucose levels) and *in vitro* (normal islet hormones, islet dysfunction)
Moffett *et al*. 2006 *Peptides*	adopted from "Asnicar *et al*. 2001 *Endocrinology*"	C57BL/6 mice (M only)	Normal chow	NSD	↑ at 20 week	N/A	N/A