Entry of CART into brain is rapid but not inhibited by excess CART or leptin

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Kastin, Abba J., and Victoria Akerstrom. Entry of CART into brain is rapid but not inhibited by excess CART or leptin. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E901–E904, 1999.—Cocaine- and amphetamine-regulated transcript (CART) is a new anorectic peptide found in the brain and periphery. It is closely associated with leptin, an anorectic agent saturably transported across the blood-brain barrier (BBB). Using multiple time-regression analysis, we found that CART has a rapid rate of entry into brain from blood. However, there was no self-inhibition with CART, even when perfused in blood-free buffer or in fasted mice, showing a lack of saturation. HPLC showed that at least 58% of the injected CART reached brain tissue in intact form, and capillary depletion with and without washout showed that the CART was not bound to endothelial cells or adherent to vascular components. There was no evidence for an efflux system out of the brain for CART. Thus CART can cross the BBB from blood to brain, but its rapid rate of entry is not inhibited by excess CART or leptin.

cocaine- and amphetamine-regulated transcript; blood-brain barrier; feeding; peptide

COCaine- AND AMPHETAMINE-REGULATED TRANSCRIPT (CART) is a satiety factor that is regulated by leptin and closely involved in leptin-mediated suppression of food intake (15). Animals lacking either circulating leptin (obese ob/ob mouse) or the leptin receptor (obese Zucker fa/ fa rat) have decreased expression of CART mRNA in the arcuate nucleus of the brain. Peripheral injection of ob/ob mice with leptin stimulates CART mRNA expression in this hypothalamic nucleus, which is intimately involved in feeding behavior, an effect unrelated to the effect of leptin on food intake (15). After peripheral injection, leptin is concentrated within the arcuate nucleus, an effect not seen after central injection. Central injection of an antibody to CART stimulates feeding (16).

Leptin is produced in the periphery, not the brain, and it is then transported by a saturable system across the blood-brain barrier (BBB) (6). Although CART is primarily produced in the brain, in the periphery it has been localized by immunohistochemistry to the adrenal medulla (14), where its mRNA is expressed (8), and to the intestine (9). By light microscopic and ultrastructural examination, CART is present in neuronal cell bodies and fibers of the myenteric plexus of the ileum (9). It is not known whether CART can cross the BBB to contribute to the central effects of CART or whether its entry is inhibited in a negative feedback action by leptin, another anorectic agent.

METHODS

Materials. Previously described methods were used for these experiments (4). Adult male albino ICR mice (Charles River, Wilmington, MA), 10 per group, weighing ~22 g were anesthetized with urethane (4 g/kg ip) and used as approved by the Institutional Animal Care and Use Committee. [Met(O)67]CART-(55—102) (human, MW 5261, Phoenix Pharmaceuticals, Mountain View, CA) was radiolabeled with 125I by the chloramine-T method and purified on a column of Sephadex G-10. Acid precipitation showed incorporation of 125I into [Met(O)67]CART-(55—102) of 93.7–97.3%. HPLC of the [Met(O)67]CART-(55—102) immediately before use showed more than 90% purity each time. The specific activity of the [Met(O)67]CART-(55—102) was ~200 Ci/mmol.

Multiple time-regression analysis of entry into the brain. The [Met(O)67]CART-(55—102) was injected in a dose of ~2 pmol/mouse [1 × 106 counts/min (cpm)] through the isolated left jugular vein along with 1 µCi/mouse of 99mTc-albumin in 200 µl of lactated Ringer solution containing 1% albumin. At various times up to 12.5 min after intravenous injection, blood was collected from a cut in the right carotid artery and the mouse was immediately decapitated. Serum and brain samples were obtained and counted in a dual-channel gamma counter. The ratio of the radioactivity of brain tissue and serum was calculated, and multiple time-regression analysis was applied to determine the relationship between the ratios and exposure time. Exposure time is the theoretical steady-state value of circulation time after the decay of the [Met(O)67]CART-(55—102) in blood is corrected. The slope of the linear portion of the regression line between brain-to-serum ratios and exposure time is Kt, the unidirectional influx constant. For [Met(O)67]CART-(55—102), the curve was linear for 12.5 min. To determine whether the entry of [Met(O)67]CART-(55—102) was saturable, self-inhibition was tested by the addition of 2 µg/mouse (380 pmol) of unlabeled [Met(O)67]CART-(55—102) to the injected solution. Cross-inhibition was tested with 2 µg/mouse (125 pmol) of leptin. A similar experiment was conducted with the addition of 5 µg/mouse of each of these compounds.

In another experiment, the entry of [Met(O)67]CART-(55—102) into 16 mice fasted for 48 h was compared with the entry in 16 mice not fasted (food and water freely available). One-half of the mice in each group were coinjected with unlabeled [Met(O)67]CART-(55—102) (5 µg/mouse). Some additional mice received leptin (5 µg/mouse). HPLC in blood and brain. Blood and brain samples were obtained 5, 10, 15, 30, and 60 min after intravenous injection of [Met(O)67]CART-(55—102). The brain was homogenized in phosphate-buffered saline with a glass homogenizer to which was added a cocktail of enzyme inhibitors (P8340, Sigma, St. Louis, MO). Our preliminary results indicated that this resulted in almost a doubling of the amount of intact [Met(O)67]CART-(55—102) recovered from brain tissue.
after it was homogenized. The enzyme inhibitors were not added to the serum samples.

After centrifugation at 5,000 g at 4°C for 10 min, the supernatant was lyophilized and rehydrated 10 min before elution on a reversed-phase C18 column. Recovery of 125I-[Met(O)67]CART-(55—102) at this step was ~93%. The gradient consisted of 0.1% trifluoroacetic acid in water, with acetonitrile increasing from 10–60% over 40 min. Flow rate on the HPLC was 1 ml/min, and 1-ml fractions were collected. Values were corrected for processing as determined by addition of 125I-[Met(O)67]CART-(55—102) to blood and homogenized brain samples of uninjected mice.

Capillary depletion with and without perfusion. The capillary depletion method was used to separate cerebral capillaries from the brain parenchyma. Each of eight mice received an intravenous injection of 125I-[Met(O)67]CART-(55—102) together with 99mTc-albumin in 200 µl of lactated Ringer-bovine serum albumin at time 0. At 10 min, four of the mice were perfused intracardially over 30 s with 20 ml of the Ringer solution, while the descending aorta was blocked and bilateral jugular veins were severed. Then the mice were decapitated, and brain samples were collected. The cerebral cortex (not containing circumventricular organs) was homogenized in a glass homogenizer in physiological buffer and mixed thoroughly with 26% dextran. An aliquot of the homogenate was centrifuged at 5,400 g for 15 min at 4°C.

The pellet, containing the capillaries, and the supernatant, representing the brain parenchymal-interstitial fluid space, were carefully separated. The ratios of radioactivity of 125I-[Met(O)67]CART-(55—102) in the supernatant (parenchyma) or pellet (capillary) over serum, corrected by subtraction of 99mTc-albumin ratios of radioactivity representing vascular space, were used to determine the 125I-[Met(O)67]CART-(55—102) in three compartments of the cortex: 1) tightly bound to vascular endothelial cells (after washout), 2) loosely associated with the vascular endothelial cells or circulating cellular elements (brain cortex before washout – after washout), and 3) in the brain parenchyma (after washout).

Perfusion in a blood-free solution. 125I-[Met(O)67]CART-(55—102) together with 99mTc-albumin was added to buffer and perfused through the left ventricle of the heart at a rate of 2 ml/min for 4 min in six anesthetized mice in which the thoracic aorta had been clamped and both jugular veins had been severed immediately before the perfusion. A 20-ml wash followed. The brain-to-perfusate ratio was corrected for albumin. Another group of five mice received unlabeled [Met(O)67]CART-(55—102), which was added to the perfusate at a concentration of 2 µg/ml. Efflux from brain. About 25,000 cpm of both 125I-[Met(O)67]CART-(55—102) and 99mTc-albumin were simultaneously injected into the brain of mice anesthetized with urethane at a site 1 mm lateral and 0.2 mm posterior to the bregma through a 1-µl Hamilton syringe (1). Mice were studied (n = 10/group) at 0, 2, 5, 10, and 20 min after injection. The 0-min value was determined in mice overdosed with anesthesia before injection, as previously explained (3). The half-time disappearance was determined from the regression line obtained from the plot of the logarithm of brain radioactivity against time.

Octanol-buffer partition coefficient. One milliliter of a 0.25 M phosphate buffer solution was added to a mixture of 125I-[Met(O)67]CART-(55—102) and 1 ml of octanol. After being vigorously mixed for 1 min and gently mixed for an additional 10 min, the two phases were separated by centrifugation at 4,000 g for 10 min. Aliquots were counted for radioactivity, and the partition coefficient was expressed as the ratio of cpm in the octanol phase to the cpm in the buffer phase.

Statistics. Groups were compared by ANOVA followed by Duncan’s multiple range test. Regression lines were determined by the least-squares method.

RESULTS

Entry into brain. The rate of entry (K1) of 125I-[Met(O)67]CART-(55—102) was 5.10 × 104 ml·g−1·min−1. Addition of 2 µg/mouse of unlabeled [Met(O)67]CART-(55—102) or leptin did not significantly change the rate of entry of the 125I-[Met(O)67]CART-(55—102). The K1 of 125I-[Met(O)67]CART-(55—102) was many times faster than that for the simultaneously injected 99mTc-albumin. Addition of excess unlabeled [Met(O)67]CART-(55—102) or unlabeled leptin did not increase the K1 of the 99mTc-albumin, indicating the lack of any disruption of the BBB. These results are shown in Fig. 1.

Similarly, the addition of a higher dose (5 µg/mouse) of unlabeled [Met(O)67]CART-(55—102) to the injection did not reduce the rate of entry of either 125I-[Met(O)67]CART-(55—102) or 99mTc-albumin. At this higher dose of unlabeled material, the intercepts for [Met(O)67]CART-(55—102) and particularly leptin were higher, indicating a larger distribution, although the slopes did not differ.

Fasting mice for 48 h did not result in any significant change in the rate of entry of 125I-[Met(O)67]CART-(55—102) into brain (K1 = 5.17 × 10−4 ml·g−1·min−1; Fig. 2). The addition of either excess (5 µg/mouse) [Met(O)67]-CART-(55—102) or leptin did not decrease K1, in either the fasted or control groups.

HPLC. In blood, the radioactivity eluting at the same position as the 125I-[Met(O)67]CART-(55—102) standard, corrected for processing, showed 92.8% was intact at 5 min. At 10 min, 80.3% was intact. In brain tissue, there was ~58% intact at these times. The percentage of intact peptide at various times up to 60 min is shown in Fig. 3. The radioactivity entering the brain as metabolites of 125I-[Met(O)67]CART-(55—102) might result in overestimation of the K1, after correction for serum degradation, and masking of saturation, if it acted at the same site of transport. The time needed for
intact $^{125}$I-[Met(O)$^{67}$]CART-(55—102) to decrease by 50% was 37.1 min for serum and 193.7 min for brain. The long half-time of intact $^{125}$I-[Met(O)$^{67}$]CART-(55—102) in the brain does not eliminate the possibility of the occurrence of more rapid metabolism with efflux of the degradation products out of the central nervous system.

Capillary depletion. Ten minutes after intravenous injection of $^{125}$I-[Met(O)$^{67}$]CART-(55—102), there was almost eight times more radioactivity in the parenchyma than bound to the capillaries ($P < 0.01$). Similarly, relatively little $^{125}$I-[Met(O)$^{67}$]CART-(55—102) was reversibly associated with the vasculature compared with that found in the brain parenchyma ($P < 0.01$). These results show that 83.4% of the injected $^{125}$I-[Met(O)$^{67}$]CART-(55—102) reached the parenchyma (Fig. 4).

Perfusion in blood-free solution. Like the results seen after a single injection into blood, perfusion of $^{125}$I-[Met(O)$^{67}$]CART-(55—102) showed much faster entry than that of $^{99m}$Tc-albumin. Addition of unlabeled [Met(O)$^{67}$]CART-(55—102) to the perfusate did not significantly change the rate of entry of either compound.

Efflux from brain. The half-time disappearance from brain was 27.4 min for $^{125}$I-[Met(O)$^{67}$]CART-(55—102) and 22.2 min for $^{99m}$Tc-albumin. The difference in the time for $^{99m}$Tc-albumin from some of our earlier experiments is probably explained by the different coordinates used for injection. Because the half-time disappearance of $^{125}$I-[Met(O)$^{67}$]CART-(55—102) is slower than that for $^{99m}$Tc-albumin, there was no obvious brain-to-blood transport system for the injected [Met(O)$^{67}$]CART-(55—102). The intercepts, however, were significantly different ($P < 0.001$), suggesting that [Met(O)$^{67}$]CART-(55—102) is retained in brain. This makes it possible that not all of the $^{125}$I-[Met(O)$^{67}$]CART-(55—102) was available for transport out of the brain so that an efflux system cannot be definitively excluded. The results are shown in Fig. 5.

Octanol-buffer partition coefficient. The octanol coefficient, calculated as the cpm in the octanol phase divided by the cpm in the buffered-saline phase, was 0.012 $\pm$ 0.001 for $^{125}$I-[Met(O)$^{67}$]CART-(55—102).

DISCUSSION

Unlike leptin, another anorectic peptide intimately involved with CART, the entry of CART into brain does...
not occur by a saturable transport mechanism. Instead, CART probably enters by simple diffusion, making it less susceptible to the regulatory function of the BBB. In this regard, the mechanism of penetration of CART across the BBB is like that of neuropeptide Y (NPY) (12), an appetite-stimulating peptide. The feeding response of NPY is completely blocked by central administration of CART-(55–102) (15). Although both peptides are found in the arcuate nucleus, their staining patterns are different (15).

A few other peptides involved in food ingestion have been found to cross the BBB by nonsaturable mechanisms. Of these, NPY (12), orexin A (13), and cyclo(His-Pro) (5), all enter the brain at rates slower than that of CART (\( K_v = 5.1 \times 10^{-4} \text{ ml} \cdot \text{g}^{-1} \cdot \text{min}^{-1} \)). Even though cyclo(His-Pro) has the slowest \( K_v \) (1.8 \( \times 10^{-4} \text{ ml} \cdot \text{g}^{-1} \cdot \text{min}^{-1} \)) of this group, its entry is sufficient to reverse ethanol-induced narcosis (5). Corticotropin-releasing hormone also is involved in the actions of leptin (11) but essentially does not enter the brain; it is the only one of these ingestive substances for which a brain-to-blood transport system has been found (18, 19).

Several physicochemical characteristics of a molecule influence its passage across the BBB. For most peptides, lipophilicity is a more important predictor of penetration than molecular weight, percent unbound peptide, total molecular charge, net charge, or absolute charge (2). More recently, hydrogen-bonding potential (7) and the cross-sectional area of the molecule oriented at a hydrophilic-hydrophobic interface (10) have been shown to be important determinants of membrane permeation. The low octanol-buffer partition coefficient for CART indicated that lipid solubility did not explain the relatively rapid entry of CART into the brain. Orexin A, which also enters the brain by transmembrane diffusion, has an octanol coefficient almost 20 times higher than that of CART even though its entry rate is only one-half as fast (13).

The lack of saturation of the entry of CART into brain was shown in several experiments, including perfusion in a blood-free medium that eliminated the possibility of binding to blood proteins. The lack of cross-inhibition by leptin also was shown in the several experiments. The lack of substantial binding to endothelial cells or adherence to vascular components was shown by capillary depletion with and without washout. HPLC showed that at least 58% of the injected CART entered the brain in intact form, although some of the degradation could have occurred after CART crossed the BBB intact. Entry of the degradation products could have resulted in overestimation of the \( K_v \).

Thus CART can readily cross the BBB. Its rapid rate of entry into the brain does not appear to be influenced by negative feedback from another anorectic agent, leptin.

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REFERENCES


