Agouti-Related Protein(83-132) Aggregates and Crosses the Blood-Brain Barrier Slowly

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Agouti-related protein (AgRP), expressed in both the periphery and the brain, can result in obesity. Its active C-terminal fragment, AgRP(83-132), was recently reported to increase feeding and antagonize alpha-melanocyte–stimulating hormone (α-MSH) and leptin. We used multiple-time regression analysis to show that the rate at which AgRP(83-132) crossed the blood-brain barrier (BBB) from the blood to the brain was very slow ($K_i = 0.6 \times 10^{-4}$ mL/g · min). Entry was not self-inhibited by excess AgRP(83-132) after either intravenous (IV) injection or perfusion in blood-free medium, indicating the absence of a saturable transport system, and was not cross-inhibited by α-MSH or leptin. Not only did AgRP(83-132) cross much slower than the saturably entering leptin, but the entry was slower than almost all other non–saturably entering endogenous peptides or neurotrophins. Nevertheless, high-performance liquid chromatography (HPLC) showed that the small amount of AgRP(83-132) crossing the BBB did so in intact form, and capillary depletion showed that it entered the brain parenchyma rather than binding to capillary endothelial cells or adhering to vascular components. There was no rapid efflux system out of the brain that might have misleadingly appeared as slow entry for AgRP(83-132). Poor lipophilicity was shown by a low octanol/buffer partition coefficient. By size-exclusion chromatography, AgRP(83-132) appeared as a 17-kd substance in both blood and buffer. Since protein was absent from the buffer, the 17-kd peak probably represented a trimer of the 5.7-kd AgRP(83-132). Capillary electrophoresis confirmed that most of the AgRP(83-132) existed as a trimer, with much smaller amounts as a dimer and monomer. Thus, although intact AgRP(83-132) can cross the BBB from the blood to the brain, its nonsaturable rate of entry is very slow, probably influenced by aggregation.

AGOUTI-RELATED PROTEIN (AgRP) is one of the newly described endogenous agents involved in the control of body weight. Its mRNA is increased in the hypothalamus of mice under conditions generally associated with leptin deficiency.[1-4] AgRP reverses the inhibition of food intake and decrease of body weight induced by alpha-melanocyte–stimulating hormone (α-MSH)[5] and leptin,[4] and its hypothalamic expression is inhibited by leptin.[2-4] AgRP also is expressed in the periphery.[1,6] The C-terminal fragment, AgRP(83-132), exerts similar actions as the full-length polypeptide, but its ability to increase feeding has been studied only after central administration.[5]

Like leptin but not several other ingestive substances, the effects of AgRP(83-132) on feeding are relatively long-lasting.[5] Unlike AgRP(83-132), which stimulates feeding, leptin inhibits feeding and is not expressed in the brain. Yet the effects of both leptin and AgRP on obesity are mediated by the brain, particularly the arcuate nucleus. Many AgRP-expressing neurons in the medial portion of the arcuate nucleus coexpress the leptin receptor.[3]

To directly reach the brain from the blood, ingestive peptides like AgRP(83-132) and polypeptides like leptin must cross the blood-brain barrier (BBB). The BBB is considered to be primarily composed of specialized endothelial cells of cerebral capillaries that lack the openings characteristic of capillaries elsewhere but are instead joined by tight junctions. The structural properties of the BBB prevent the free exchange of ingestive substances between the blood and brain. Nonetheless, specific transport systems are present at the BBB for some bioactive peptides and polypeptides.[7,8]

Leptin crosses the BBB rapidly by a saturable transport mechanism.[9] We studied whether AgRP(83-132), which may have possible therapeutic use in the treatment of anorexic conditions, also crosses the BBB from the blood to the brain.

MATERIALS AND METHODS

Multiple-Time Regression Analysis of Entry Into the Brain

Adult male albino ICR mice (Charles River, Wilmington, MA; 10 per group) weighing about 22 g were anesthetized with urethane (4 g/kg intraperitoneally). AgRP(83-132)NH_2 (human; Phoenix Pharmaceuticals, Mountain View, CA) was radiolabeled with ^{125}I by the chloramine-T method and purified on a column of Sephadex G-10. Acid precipitation showed 98.3% incorporation of ^{125}I into AgRP(83-132). High-performance liquid chromatography (HPLC) of ^{125}I-AgRP(83-132) showed 96.6% purity. The specific activity of ^{125}I-AgRP(83-132) was about 200 Ci/mmol.

^{125}I-AgRP(83-132) was injected at a dose of about 2.5 pmol per mouse (1×10^6 cpm) via the isolated left jugular vein along with 1 μCi per mouse of ^{99m}Tc-albumin in 200 μL lactated Ringer's solution containing 1% albumin. At 2, 5, 10, 15, 30, 45, and 60 minutes after intravenous (IV) 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 analyzed 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 ratio and the exposure time.

There was a linear relationship between the ratio of tissue/serum radioactivity and exposure time, which represents the theoretical steady-state value for circulation time after correction for decay of ^{125}I-AgRP(83-132) in blood.[10] The slope of this regression line represents the influx rate (K_i) of AgRP(83-132). To determine whether the entry of ^{125}I-AgRP(83-132) was saturable, self-inhibition was tested by

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the addition of 5 μg per mouse (881 pmol) unlabeled AgRP(83-132) to the injected solution. Cross-saturation was tested with 5 μg per mouse (313 pmol) leptin and 5 μg per mouse α-MSH (3 nmol).

HPLC in Blood and Brain

Blood and brain samples were obtained 5, 15, 30, 45, and 60 minutes after injection of ^{125}I-AgRP(83-132). The brain sample was homogenized in phosphate-buffered saline with a glass homogenizer. A cocktail of enzyme inhibitors (P8340; Sigma, St Louis, MO) was added to the brain but not to the serum samples. After centrifugation, the supernatant was lyophilized and rehydrated 10 minutes before elution (10% to 60% acetonitrile in water with 0.1% trifluoroacetic acid) on a C18 column. Values were corrected for processing as determined by the addition of ^{125}I-AgRP(83-132) to the blood and homogenized brain samples of uninjected mice.

Capillary Depletion With and Without Perfusion

Capillary depletion with washout was used to separate cerebral capillaries and vascular components from brain parenchyma. Each of 8 mice received an IV injection of about 6×10^6 cpm ^{125}I-AgRP(83-132) together with ^{99m}Tc-albumin in 200 μL lactated Ringer's/1% bovine serum albumin at time 0. At 10 minutes, 4 of the mice were perfused intracardially over 30 seconds with 20 mL Ringer's solution while the descending aorta was blocked and the bilateral jugular veins were severed. The remaining 4 mice did not undergo the washout procedure. All 8 mice were then decapitated and brain samples were collected. The cerebral cortex (not containing circumventricular organs) was homogenized in glass with physiologic buffer and mixed thoroughly with 26% dextran. An aliquot of the homogenate was centrifuged at $5,400 \times g$ for 15 minutes at 4°C.

The pellet, containing the capillaries, was carefully separated from the supernatant, representing the brain parenchymal/interstitial fluid space. The ratio of the radioactivity of ^{125}I-AgRP(83-132) in the supernatant (parenchyma) or pellet (capillary) over serum, corrected by subtraction of the ^{99m}Tc-albumin ratio of radioactivity representing the vascular space, was used to determine ^{125}I-AgRP(83-132) in 3 compartments: (1) tightly bound to vascular endothelial cells (after washout), (2) loosely associated with vascular endothelial cells or circulating cellular elements (brain cortex before washout minus after washout), and (3) in the brain parenchyma (after washout).

Perfusion in a Blood-Free Solution

^{125}I-AgRP(83-132) and ^{99m}Tc-albumin were added to the buffer and perfused through the left ventricle of the heart at a rate of 2 mL/min for 4 minutes in 6 anesthetized mice in which the thoracic aorta was clamped and both jugular veins were severed immediately before perfusion. A 20-mL wash followed. The brain to perfusate ratio was corrected for albumin. Another group of 6 mice received unlabeled AgRP(83-132) added to the perfusate at a concentration of 2 μg/mL (352 pmol/mL).

Efflux From Brain

About 25,000 cpm of both ^{125}I-AgRP(83-132) and ^{99m}Tc-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.[11] Mice were studied ($n = 5$ per group) at 0, 2, 5, 10, and 20 minutes after injection. The value at 0 minutes was determined in mice overdosed with anesthesia before injection, as previously explained.[12] 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

To a mixture of ^{125}I-AgRP(83-132) and 1 mL octanol was added 1 mL 0.25-mol/L phosphate buffer solution. After vigorous mixing for 1 minute and gentle mixing for an additional 10 minutes, the two phases were separated by centrifugation at $4,000 \times g$ for 10 minutes. Aliquots were analyzed for radioactivity and the partition coefficient expressed as the ratio of cpm in the octanol phase to cpm in the buffer phase.

Statistics

Groups were compared by ANOVA followed by Duncan's multiple-comparison test. Regression lines were determined by the least-squares method, and differences between the slopes were compared by GraphPad Prism statistical software (GraphPad Software, San Diego, CA).

Size-Exclusion Chromatography

The possibility of aggregation or binding to serum proteins was examined on a BioSep SEC S-2000 HPLC column (Phenomenex, Torrance, CA). ^{125}I-AgRP(83-132) was incubated in serum at 37°C for 15 minutes, filtered on a 0.45-μm filter to remove gross impurities, centrifuged, and added to the prewashed column equilibrated in 0.1 mol/L phosphate buffer, pH 6.8. The flow rate was 0.2 mL/min with an isocratic gradient. Fractions of 1 mL/min were collected. The marker for 670 kd was thyroglobulin; for 150 kd, IgG; for 44 kd, ovalbumin; for 17 kd, myoglobin; for 5.8 kd, insulin; and for 0.24 kd, uridine.

Capillary Electrophoresis

Analysis was performed with a Beckman (Fullerton, CA) SDS 14-200 kit on a Beckman P/ACE 5510 capillary electrophoresis system equipped with a diode array detector. The length of the SDS-coated capillary was 27 cm. The voltage was 8.1 kV (300 V/cm) and the temperature was 20°C. The procedure consisted of a 1-minute rinse with 1 mol/L HCl, a 3-minute rinse with gel buffer, pressure injection, and separation over 16 minutes. The molecular weight of each substance was calculated from the relative migration time determined with a Beckman protein mix standard calibration curve.

RESULTS

Entry Into Brain

The K_i of ^{125}I-AgRP(83-132) was 0.57×10^{-4} mL/g · min. The addition of excess unlabeled AgRP(83-132) ($K_i = 0.61 \times 10^{-4}$ mL/g · min), leptin ($K_i = 0.88 \times 10^{-4}$ mL/g · min), or α-MSH ($K_i = 0.48 \times 10^{-4}$ mL/g · min) did not significantly change the rate of entry of ^{125}I-AgRP(83-132). Moreover, the addition of these excess unlabeled substances did not increase the K_i of ^{99m}Tc-albumin, indicating a lack of disruption of the BBB. The results are shown in Fig 1.

HPLC

In the blood, radioactivity eluting at the same position as the ^{125}I-AgRP(83-132) standard, corrected for processing, showed that 43.7% was still intact at 60 minutes. In brain tissue, 52.5% remained intact at 60 minutes. The percentage of intact peptide at the various times sampled up to 60 minutes is shown in Fig 2. The corrected half-time disappearance calculated for the serum was 81 minutes, and for the brain, 289 minutes.

Capillary Depletion

Ten minutes after IV injection of ^{125}I-AgRP(83-132), there was a significant difference ($F(3,11) = 37.1$, $P < .01$) in radio-

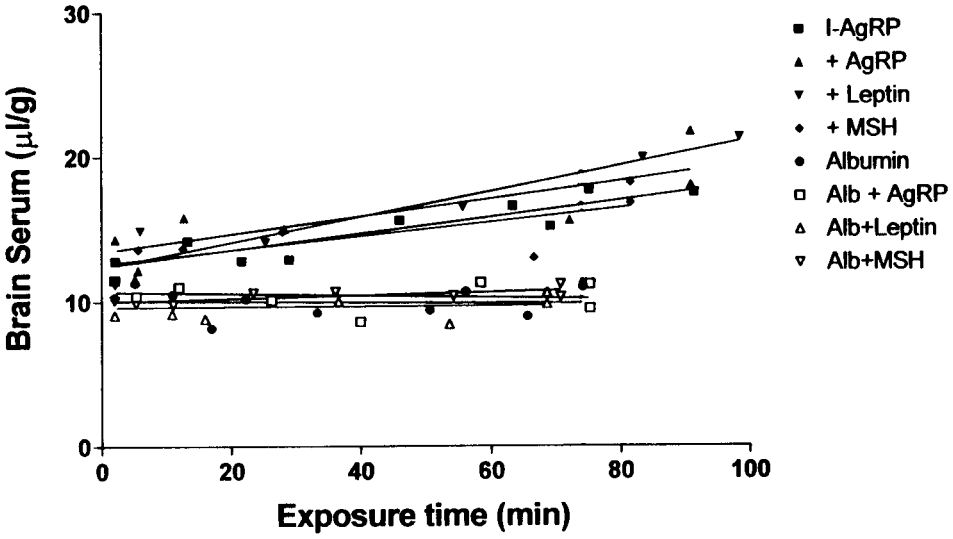

Fig 1. Blood-to-brain influx of ^{125}I-AgRP(83-132) (I-AgRP) and ^{99m}Tc-albumin (Alb) with and without the addition of 5 μg/mouse of unlabeled AgRP-(83-132), leptin, or α-MSH. Exposure time represents experimental time corrected for disappearance in blood. Entry of ^{125}I-AgRP(83-132) was not significantly inhibited by excess AgRP(83-132), leptin, or α-MSH.

activity in the different compartments of the brain. More than 5-fold greater radioactivity was found in the parenchyma versus the capillaries ($P < .001$). More than 10×10^6 cpm reached the parenchyma before washout; the washout removed only a few hundred cpm. This shows the relatively small amount of ^{125}I-AgRP(83-132) that is reversibly associated with the vasculature as compared with the brain parenchyma ($P < .001$). About 83% of radioactivity measured in the brain was present in the parenchyma. Figure 3 shows the mean ± SEM in these compartments.

Perfusion in Blood-Free Solution

Perfusion of ^{125}I-AgRP(83-132) in blood-free buffer together with excess unlabeled AgRP(83-132) did not show any significant reduction in the rate of entry of the labeled peptide.

Efflux From Brain

The half-time disappearance of ^{125}I-AgRP(83-132) from the brain was negligibly slower than the half-time disappearance of ^{99m}Tc-albumin. This suggests the absence of a brain-to-blood transport system for ^{125}I-AgRP(83-132). These results are shown in Fig 4.

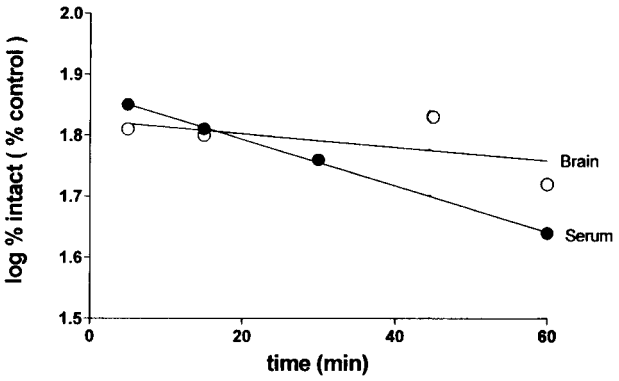

Fig 2. Disappearance of ^{125}I-AgRP(83-132) from brain and serum after IV injection. HPLC was used to determine intact peptide.

Octanol/Buffer Partition Coefficient

The octanol coefficient, calculated as cpm in the octanol phase divided by cpm in the buffered saline phase, was 0.011 ± 0.0001 for ^{125}I-AgRP(83-132).

Size-Exclusion Chromatography

Incubation of ^{125}I-AgRP(83-132), with a molecular weight of 5.7 kd (5,676), in serum for 15 minutes resulted in most of the material eluting at a peak corresponding to that of 17 kd. This was the same position as the control incubated in protein-free buffer. The results are shown in Fig 5.

Capillary Electrophoresis

About 70% of AgRP(83-132) eluted at a position corresponding to 17 kd. A smaller peak of about 26% corresponded to the dimer at about 11.5 kd, and the smallest peak of about 4% corresponded to the monomer at 5.7 kd. The electrophoretogram is shown in Fig 6.

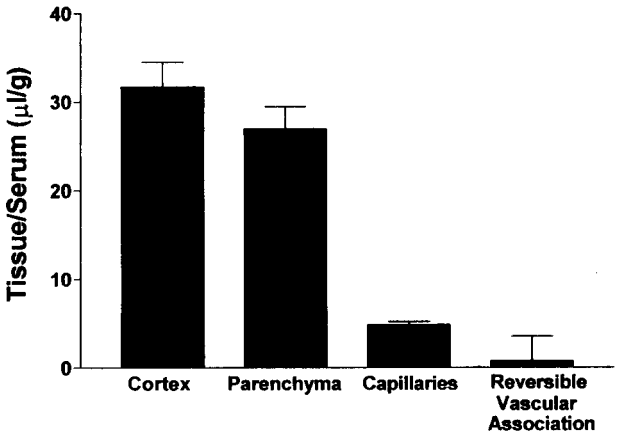

Fig 3. Capillary depletion study showing ^{125}I-AgRP(83-132) in various compartments of the brain cortical fraction. About 83% of the injected radioactivity reached the parenchyma of the brain.

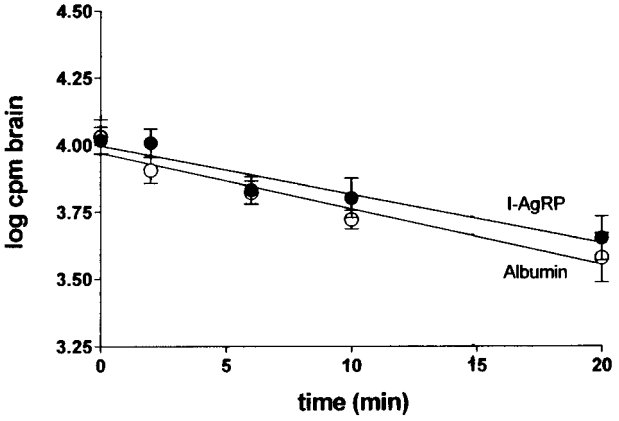

Fig 4. Efflux of ^{125}I-AgRP(83-132) and ^{99m}Tc-albumin from brain after central injection.

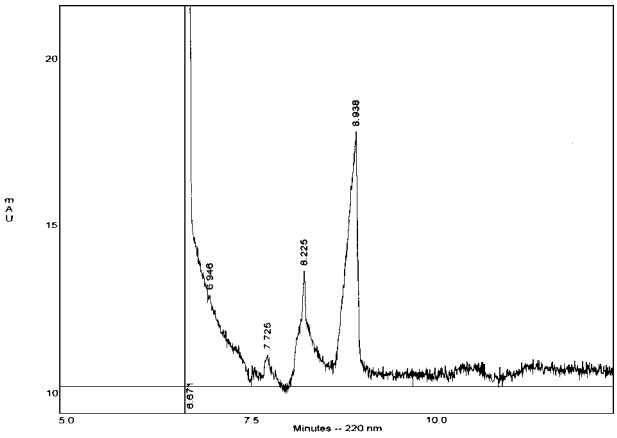

Fig 6. Capillary electrophoresis of AgRP(83-132). Most of the peptide eluted as a 17-kd trimer, with smaller amounts as a dimer and a monomer.

DISCUSSION

The crossing of the BBB by AgRP(83-132) was not self-inhibited, even with perfusion in blood-free medium, and was not cross-inhibited by α-MSH or leptin, two ingestive substances with which AgRP interacts.[2-5,13] BBB penetration by AgRP(83-132) was much slower than that of leptin. Since leptin crosses the BBB by a saturable transport system[9] and AgRP(83-132) does not, a difference in the penetration rate may have been expected. Perhaps the effects in the brain of AgRP produced centrally predominate over those produced peripherally, subject to feedback control by the peripherally produced leptin crossing the BBB. Moreover, there are many other processes by which a peptide in the periphery can affect the brain besides direct penetration of the BBB in intact form.[14]

The K_i for AgRP(83-132) of 0.6×10^{-4} mL/g · min is slower than that found for most of the peptides we have tested by this method. This includes several other ingestive peptides such as amylin,[15,16] cyclo(His-Pro),[17] neuropeptide Y,[18] orexin A,[19] and cocaine- and amphetamine-regulated transcript [CART(55-102)].[20] However, corticotropin-releasing hormone[21] and melanin-concentrating hormone[22] do not cross the BBB from the blood faster than albumin. The entry rate of endogenous ingestive substances therefore appears to be unrelated to whether they increase or decrease feeding.

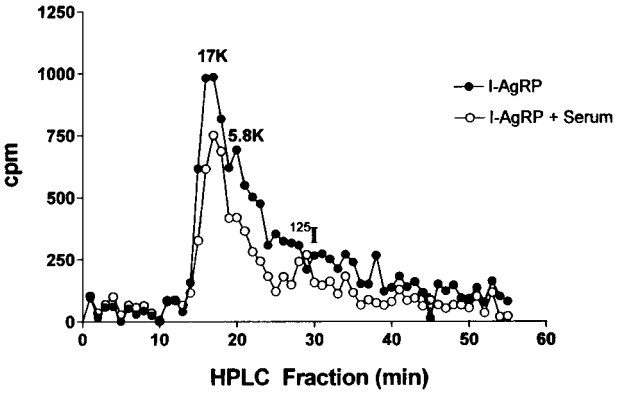

Fig 5. Size-exclusion chromatogram of ^{125}I-AgRP(83-132) in serum and buffer. Most of the material eluted at 17 kd, consistent with a trimer of the 5.7-kd peptide.

Consistent with its relatively long action in increasing feeding behavior,[5] AgRP(83-132) was remarkably stable in blood and brain. HPLC showed that almost half of the ^{125}I-AgRP(83-132) found 1 hour after IV injection remained intact. Moreover, capillary depletion with washout showed that most of the IV injected ^{125}I-AgRP(83-132) detected in brain reached the parenchyma. Relatively little was bound to the endothelial cells comprising the BBB or loosely adherent to vascular elements.

Since the amount of peripherally administered AgRP(83-132) measured in the brain was low, it was possible that the injected peptide was pumped out of the brain so fast that it might have misleadingly appeared as if little had entered. However, AgRP(83-132) injected intracerebroventricularly left the brain at the same rate as albumin, reflecting the normal rate of absorption of cerebrospinal fluid.

Of the many physicochemical properties involved in simple diffusion across the BBB, lipophilicity is one of the most important.[23] The low octanol coefficient for AgRP(83-132) is therefore consistent with its slow rate of entry. Although CART(55-102) also crosses the BBB nonsaturably with a similar octanol coefficient, its K_i is more than 6 times faster.[20] This suggests that other physicochemical properties are involved in the slow, nonsaturable entry of AgRP(83-132) into the brain.

Size-exclusion chromatography showed that ^{125}I-AgRP(83-132) was present in the blood as a larger molecule. This would have indicated protein binding as the most likely explanation for the slow rate of entry, except that ^{125}I-AgRP(83-132) also was present in protein-free buffer in the same 17-kd form. Moreover, 17 kd is almost exactly 3 times the size of the AgRP(83-132) monomer (5.7 kd), making aggregation the more likely explanation, as we previously observed for delta sleep-inducing peptide and other peptides.[24]

The more sensitive technique of capillary electrophoresis was then applied to determine the various forms of AgRP(83-132). It was able to differentiate a primary peak (70%) consistent with a

trimer of AgRP(83-132), as well as smaller amounts representing the dimer (26%) and monomer (4%) forms of this peptide.

Peptides are seldom checked for aggregation. The usual techniques of amino acid analysis, sequencing, mass spectroscopy, and HPLC would miss aggregation in some cases because of the preparative steps intrinsic to the procedure. Even if detected, the degree of aggregation, like that of conformation, probably varies with the solution in which it is examined. However, for AgRP(83-132), aggregation as a trimer seems to predominate in several media, including serum.

On the one hand, it seems reasonable to assume that the circulation of AgRP(83-132) in aggregated form would contribute to its slow rate of entry. Interference with entry could occur by several mechanisms, ranging from an unfavorable conformation of the trimer to a covering of hydrogen-bonding groups. On the other hand, nerve growth factor (NGF) enters the brain faster than its much smaller βNGF subunit.[25] Moreover, brain-derived neurotrophic factor also probably exists in aggregated form,[26] but it crosses the BBB twice as fast as the smaller AgR P(83-132) and does so by a saturable transport system that is self-inhibitable.

Regardless, AgRP(83-132) in the blood is able to cross the BBB to reach brain cells in intact form. Aggregation probably contributes to its slow rate of entry.

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