Orexin A but Not Orexin B Rapidly Enters Brain from Blood by Simple Diffusion

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ABSTRACT

We determined the ability of orexin A and orexin B, recently discovered endogenous appetite enhancers, to cross the blood-brain barrier (BBB) of mice. Multiple time-regression analysis showed that an i.v. bolus of 125I-orexin A rapidly entered the brain from the blood, with an influx rate (Kᵢ = 2.5 ± 0.3 × 10⁻⁶ ml/g-min) many times faster than that of the 99mTc-albumin control. This relatively rapid rate of entry was not reduced by administration of excess orexin A (or leptin) or by fasting for 22 h, even when penetration into only the hypothalamus was measured. Lack of saturability also was shown by perfusion in blood-free buffer. HPLC revealed that most of the injected 125I-orexin A reached the brain as intact peptide. Capillary depletion studies showed that the administered peptide did not remain bound to the endothelial cells comprising the BBB but reached the brain parenchyma. Efflux of 125I-orexin A from the brain occurred at the same rate as 99mTc-albumin. The octanol-to-buffer partition coefficient of 0.232 showed that orexin A was highly lipophilic, whereas the value for orexin B was only 0.030. Orexin B, moreover, was rapidly degraded in blood, so no 125I-orexin B could be detected in intact form in brain when injected peripherally. Thus, although orexin B is rapidly metabolized in blood and has low lipophilicity, orexin A rapidly crosses the BBB from blood to reach brain tissue by the process of simple diffusion.

The orexins were named for the Greek word for appetite (Sakurai et al., 1998), whereas anorexia means the absence of appetite. Administration of the orexin peptides centrally has been found to stimulate appetite (Sakurai et al., 1998).

The orexins were discovered during a search for peptides that activate orphan receptors whose amino acid sequences show the structural hallmarks of cell surface receptors for G proteins (Barinaga, 1998; Sakurai et al., 1998; Wolf, 1998). HPLC of rat brain extracts resulted in a major peak of orexin A, found to consist of 33 amino acids (molecular weight, 3562) and a minor peak of orexin B containing 28 amino acids of which 46% are identical with orexin A. The orexin A receptor in brain, deduced from the cDNA of the G protein-coupled cell surface receptor, shows 26% identity with neuropeptide Y but has a more restricted distribution. Only a small amount of prepro-orexin mRNA is found in the periphery. The orexin peptides are primarily located in the lateral hypothalamus but not in the ventromedial hypothalamus, which is involved with many other feeding peptides.

To assess the therapeutic potential of the orexins for the treatment of anorexia, it would be helpful to know their ability to cross the blood-brain barrier (BBB) from the periphery. Accordingly, the rate of entry into brain of i.v. 125I-labeled orexin A and orexin B was quantified by multiple time-regression analysis. HPLC was used to determine whether the 125I-orexins penetrated the BBB in intact form. Capillary depletion was used to determine whether most of the 125I-orexin reaching the brain was in the parenchyma or was bound to the endothelial cells comprising the BBB. 99mTc-labeled albumin was injected together with the 125I-orexin to reflect the vascular space and any disruption of the BBB. The octanol-to-buffer ratio was used to measure the lipophilicity of the orexins. Efflux from the brain and entry by a blood-free i.v. perfusion also were tested.

Materials and Methods

Multiple Time-Regression Analysis of Entry into Brain.

Adult male albino ICR mice (Charles River, Wilmington, MA) (10 per group and weighing about 22 g) were anesthetized with urethane (4 g/kg i.p.). Orexin 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 orexin of 95.7% to 99.6% for the iodings used in this study. HPLC of the 125I-orexin immediately before use showed more than 90% purity each time. The specific activity of 125I-orexin A was 126 Ci/mmol, and that of 125I-orexin B was 102 Ci/mmol.

The 125I-orexin was injected in a dose of 6 pmol/mouse (2 × 10⁶ cpm) through the isolated left jugular vein along with 2 μCi/mouse of 99mTc-albumin in 200 μl of lactated Ringer’s solution containing 1%...
albumin. At 2, 3, 4, 5, 6, 8, 10, 20, and 30 min after i.v. injection, blood was collected from a cut in the right carotid artery, and then the mouse was immediately decapitated. Serum and brain samples were obtained and counted in a dual channel γ-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.

All procedures were performed in glass rather than the polypropylene tubes usually used for peptides. Our preliminary studies indicated a large loss of recoverable material in the plastic but surprisingly little loss in glass.

Exposure time is the theoretical steady-state value of circulation time after the decay of the $^{125}$I-orexin in blood is corrected. Because peptides have slow penetration into the brain, a linear relationship for orexin was present between the tissue-serum radioactivity ratio and exposure time. The slope of this regression line represents the influx rate ($K_i$) of orexin. To determine whether the entry of $^{125}$I-orexin was saturable, self-inhibition was tested by the addition of 2 µg/mouse (560 pmol for orexin A) of unlabeled material in the injected solution (Banks and Kastin, 1993). The cyclic 33-amino-acid orexin A with two intramolecular disulfide bonds and a protected (pyroGlu) N terminus (completely conserved for mouse, rat, cow, and human) and the linear 28-amino-acid orexin B (mouse and rat) were purchased from Peptides International (Louisville, KY).

In another experiment, the saturable entry 5 min after the injection of $^{125}$I-orexin A into the hypothalamus of 24 mice fasted for 22 h was compared with the entry in 24 mice that were not fasted. Because only hypothalamus was taken in this experiment, the tissue was pooled from four mice each. This resulted in four groups consisting of three groups of pooled hypothalami: $^{125}$I-orexin A in fasted mice, $^{125}$I-orexin A plus 2 µg of unlabeled orexin A in fasted mice, $^{125}$I-orexin A in fed mice, and $^{125}$I-orexin A plus 2 µg of unlabeled orexin A in fed mice.

Octanol/Buffer Partition Coefficient as a Measure of Lipophilicity. To a mixture of $^{125}$I-orexin and 1 ml of octanol, we added 1 ml of a 0.25 M phosphate buffer solution. After vigorous mixing for 1 min and gentle mixing for an additional 10 min, the two phases were separated by centrifugation at 4000g 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.

HPLC of Orexin in Blood and Brain. Blood and brain samples were obtained after the injection of $^{125}$I-orexin A or $^{125}$I-orexin B. The pooled brain was homogenized in PBS with a glass homogenizer. After centrifugation, the supernatant was lyophilized and rehydrated 10 min before elution (10–60% acetonitrile in water with 0.1% trifluoroacetic acid) on a reversed phase C-18 column. Values were corrected for processing as determined by the addition of $^{125}$I-orexin to blood and brain samples from uninjected mice.

Capillary Depletion. The capillary depletion method was used to separate cerebral capillaries from the parenchyma. Each mouse received an i.v. injection of $^{125}$I-orexin A and $^{99m}$Tc-albumin in 200 µl of lactated Ringer’s/BSA at time 0. At 5 min, the mice were decapitated, and brain samples were collected. The cerebral cortex was homogenized in a glass homogenizer in physiological buffer and mixed thoroughly with 26% dextran to yield a final concentration of dextran slightly over 15.5%, which is necessary for proper separation of the vasculature from the brain parenchyma. An aliquot of the homogenate was centrifuged at 5400g 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 $^{125}$I-orexin A in the supernatant (parenchyma) or pellet (capillary) to that in serum, corrected by subtraction of $^{99m}$Tc-albumin ratios of radioactivity representing vascular space, were used to determine the $^{125}$I-orexin A retained bound to endothelial cells and that crossing the BBB to arrive at the parenchyma of the brain tissue.

**Results**

**Entry into Brain.** The rate of entry ($K_i$) of $^{125}$I-orexin A was $3.091 \pm 0.434 \times 10^{-4} \text{ ml/g/min}$. The addition of 2 µg/mouse of excess unlabeled orexin A did not significantly decrease the rate of entry ($K_i = 2.537 \pm 0.088 \times 10^{-4} \text{ ml/g/min}$). The results are shown in Fig. 1. Leptin (2 µg/mouse) also did not reduce the $K_i$ value ($2.37 \times 10^{-4} \text{ ml/g/min}$ for the $^{125}$I-orexin A alone used in a separate experiment and the identical $K_i$ value for $^{125}$I-orexin A plus 2 µg of leptin). A total of three experiments were performed in which the $K_i$ value of $^{125}$I-orexin A was determined after i.v. injection; the mean was $2.52 \pm 0.29 \times 10^{-4} \text{ ml/g/min}$.

The $K_i$ value of $^{125}$I-orexin A was many times faster than that for the simultaneously injected $^{99m}$Tc-albumin (0.096 ± 0.229 \times 10^{-4} \text{ ml/g/min}). The addition of unlabeled orexin did not disrupt the BBB to significantly increase the $K_i$ value of the $^{99m}$Tc-albumin (Fig. 1).

The $K_i$ value for $^{125}$I-orexin B initially appeared to be about $50 \times 10^{-4} \text{ ml/g/min}$, but HPLC showed that none of the radioactivity in the brain remained attached to the peptide.

**Perfusion in a Blood-Free Solution.** Next, $^{125}$I-orexin A or $^{125}$I-orexin B together with $^{99m}$Tc-albumin was added to buffer based on the method of Zlokovic et al. (1986) modified for use in mice. Another group of mice received unlabeled orexin A or orexin B, which was added to the perfusate at a concentration of 5.0 µg/ml. This was perfused through the left ventricle of the heart at a rate of 2 ml/min for 2 min in anesthetized mice in which the thoracic aorta had been clamped and both jugular veins severed immediately before the perfusion. A 20-ml wash followed. The brain-to-perfusate ratio was corrected for albumin.

**Efflux of Orexin from Brain.** About 25,000 cpm of both $^{125}$I-orexin A 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 26-gauge needle with all except the last 2.5 mm covered with polyethylene tubing to ensure a constant depth of penetration (Banks et al., 1997). Mice were studied ($n = 5$ 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 described (Banks and Kastin, 1989). The half-time disappearance was determined from the regression line obtained from the plot of the logarithm of brain radioactivity against time.

![Fig. 1. Blood-to-brain influx of $^{125}$I-orexin A and $^{99m}$Tc-albumin (Alb), with and without the addition of 2 µg/mouse unlabeled orexin A, plotted against exposure time (Exp t). The entry of $^{125}$I-orexin A was much faster than that of $^{99m}$Tc-albumin but was not significantly inhibited by excess orexin A, indicating a lack of saturation.](https://example.com/fig1.png)
Inclusion of 2 µg of unlabeled orexin B or leptin in the i.v.
injection did not change the rate of entry of the degradation
product that remained attached to the 125I.

For the experiment involving fasting, ANOVA showed no
significant difference among the four groups (Fig. 2). Fasting
did not change the rate of entry of 125I-orexin A into hypo-
thalamic tissue of mice without food for 22 h. The addition of
2 µg of orexin A did not inhibit entry in either the fasted or
fed groups, again showing the absence of a saturable system.

Octanol/Buffer Partition Coefficient. The octanol coef-
ficient, calculated as the cpm in the octanol phase divided by
the cpm in the buffered saline phase, was 0.232 ± 0.020 for
125I-orexin A. For 125I-orexin B, it was much lower: 0.030 ±
0.002.

HPLC for 125I-Orexin. In blood, the percent of radioac-
tivity eluting at the same position as the 125I-orexin stan-
dards, corrected for processing, is shown at various times
after i.v. injection in Fig. 3. The disappearance curves for the
intact orexins, calculated on a linear model, were signifi-
cantly (p < .01) different, revealing a half-time disappear-
ance of 4.40 min for orexin B (goodness of fit, r² = 0.93) and
30.7 min for orexin A (r² = 0.89).

In brain, 89.7% of the radioactive material, corrected for
processing, was intact 125I-orexin A at 2 min. By contrast, no
125I-orexin B remained in intact form in brain at that time
(Fig. 4).

Perfusion in Blood-Free Buffer. To eliminate the pos-
sibility that the lack of self-inhibition in the in vivo study was
caused by interference from blood components, in situ perfu-
sion in physiological buffer was used. The brain-to-perfusate
ratio of the orexins with and without the inclusion of 5 µg/ml
of their respective unlabeled orexin in the blood-free perfu-
sate did not show any statistically significant difference by
ANOVA.

HPLC of brain tissue after the 2-min perfusion in blood-
free medium showed that 18.9% of the radioactivity repre-
sented intact 125I-orexin B. More 125I-orexin B obviously
reached the brain after perfusion in buffer than after i.v. injection in blood, a condition in which no 125I-orexin B
was found intact in brain. By contrast, after 2-min perfusion,
87.3% of the 125I-orexin A was intact. More 125I-orexin A
was found intact in brain than 125I-orexin B after either i.v.
injection or perfusion, but there was no significant difference
in the amount of intact orexin A reaching the brain via either
method (Fig. 4).

Capillary Depletion. At 5 min after the i.v. injection of
125I-orexin A, there was significantly (p < .01) more 125I-
orexin A in the parenchyma than was bound to the capillar-
ies.

Efflux from Brain. The slightly slower half-time disap-
pearance of 125I-orexin A from brain was not significantly
different from the half-time disappearance of 99mTc-albumin
(Fig. 5). This indicates the lack of a brain-to-blood transport
system.

Discussion
Most endogenous peptides and polypeptides known to af-
fect appetite exert an inhibitory effect. This necessitates ex-
periments proving that the suppression of food ingestion is a
direct effect on appetite rather than an indirect toxic effect.
Such procedures are not required if the peptide increases the
intake of food. The recently described orexins enhance appe-
tite (Sakurai et al., 1998).

The potential of an appetite enhancer to pharmacologically
affect food ingestion in patients is great. On the one hand, it
could be tried in conditions such as anorexia nervosa, a
disease notoriously resistant to therapeutic manipulation, or

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**Fig. 2.** Entry into hypothalamic tissue of 125I-orexin A with and without
2 µg/mouse unlabeled orexin A in mice fasted for 22 h and their fed
controls. ANOVA showed no statistical significance.

**Fig. 3.** Disappearance from the blood of 125I-orexin A and 125I-orexin B
after i.v. injection. HPLC was used to determine intact peptides.

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**Fig. 4.** Comparison of the entry of intact 125I-orexin A and 125I-orexin B
into brain when administered i.v. and by perfusion in blood-free buffer.
HPLC was used to determine intact peptides.
the anorexia associated with many disease states, including AIDS wasting syndrome, and sometimes even their treatments (e.g., chemotherapy). On the other hand, analogs could be devised that could inhibit the action of orexin so as to be of possible benefit in obesity. Perhaps enzymatic inhibition could be used to increase bioavailability. Regardless, to represent practical therapy, these compounds should be effective when administered peripherally. Of the many ways by which a peptide like orexin can exert its central effects after peripheral administration, the simplest would be to directly cross the BBB.

The two main mechanisms by which a peptide enters the brain from the blood are by a transport system or by simple diffusion. A transport system across the BBB for the orexins is very unlikely because of the lack of saturability at the doses tested, even with a blood-free perfusion. Many factors influence the diffusion of peptides across the BBB, especially hydrogen bonding (Chikhale et al., 1994) and lipophilicity (Banks and Kastin, 1985). Our results show that orexin A (but not orexin B) is highly lipophilic, a characteristic that contributes to its rapid entry into the brain.

The rate of entry into the brain of $^{125}$I-orexin A ($K_i = 3.1 \times 10^{-4}$ ml/g/min) is among of the fastest found for nonsaturable penetration of the BBB by a peptide or polypeptide. This is consistent with its high lipophilicity as reflected in an octanol/buffer partition coefficient of 0.232. The $K_i$ value for orexin A is in the general range found for the entry of leptin (Banks et al., 1996) and insulin (Banks et al., 1997), both of which are larger than the orexins and both of which readily cross the BBB by saturable transport systems. The octanol/buffer partition coefficient for insulin (0.0215), however, is about 10 times lower than that of orexin A.

The crossing of the BBB by $^{125}$I-orexin was not saturable, as shown by the lack of a decrease in its rate of entry after the simultaneous administration of excess unlabeled orexin. No inhibition of the penetration of the BBB was found in four different groups of mice: when $^{125}$I-orexin was injected i.v. and whole brain was measured, when $^{125}$I-orexin A was injected i.v. and only hypothalamus was measured, when $^{125}$I-orexin A was injected i.v. and only hypothalamus was measured in fasted animals, and when $^{125}$I-orexin A was administered by perfusion. Entry of the $^{99m}$Tc-albumin control also was not affected by excess orexin, showing that orexin did not change the general permeability of the BBB or the vascular space.

Lack of saturation after i.v. bolus injection might be misleading if there was a transport system with a very high capacity or if the injected material was bound to circulating proteins. The use of a blood-free perfusate system eliminated any influence of blood components as an explanation for the absence of saturable entry of $^{125}$I-orexin. The addition of excess unlabeled orexin did not inhibit the entry of $^{125}$I-orexin from the perfusate. Unlike the absence of detectable entry of $^{125}$I-orexin B after i.v. injection, a small amount of $^{125}$I-orexin B could be measured in intact form in brain after perfusion in the blood-free buffer.

HPLC established for $^{125}$I-orexin A that most of the radioactivity found in the brain after injection into blood represented intact peptide rather than any degradation products retaining the radioactive $^{125}$I label. Its relative stability in blood was emphasized by the similar percentage of intact peptide reaching the brain by perfusion. In contrast, HPLC showed essentially no intact $^{125}$I-orexin B reaching the brain when injected into blood, where it was shown to be much more rapidly degraded than orexin A. Even when perfused in blood-free medium, much less $^{125}$I-orexin B entered the brain intact than did $^{125}$I-orexin A. Sakurai et al. (1998) found that the effects of orexin B on food intake were not as long-lasting as those of orexin A, perhaps related to structural differences making orexin A more resistant to inactivating peptidases.

The brain tissue obtained for measurement of radioactivity includes the presence of some cerebral capillaries. The $^{125}$I-orexin A could have been tightly bound to the endothelial cells of the capillaries, making it appear as if it entered brain cells. When the capillaries were separated from brain, significantly more $^{125}$I-orexin A was found in the parenchyma than was bound to capillaries.

The orexins were isolated from brain tissue (Sakurai et al., 1998). If a brain-to-blood transport system existed for orexin A, this might explain the nonsignificant slight increase in entry of $^{125}$I-orexin A into the hypothalamus of fed mice after the coadministration of excess unlabeled orexin A. In such an unexpected circumstance, the excess peptide could have entered the hypothalamus, where it might have inhibited a possible transport system for $^{125}$I-orexin A out of the brain. This would have resulted in greater retention of $^{125}$I-orexin A in the brain tissue that was counted after i.v. injection. Facilitated transport of orexin A out of the brain, however, was not found. The similar rates of exit for $^{99m}$Tc-albumin and $^{125}$I-orexin A show that normal bulk flow for reabsorption of cerebrospinal fluid explains the brain-to-blood egress of orexin A.

Thus, the results show that orexin B has low lipophilicity and is rapidly metabolized in blood, effectively preventing any substantial entry into brain after i.v. injection. In contrast, orexin A can rapidly cross the BBB to reach the brain from blood. Although no saturable transport system was found for orexin A, its fast rate of entry is probably explained by simple diffusion facilitated by a high degree of lipophilicity.

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References


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