Permeability of the blood–brain barrier to a novel satiety molecule nesfatin-1

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1. Introduction

Food consumption and energy expenditure are regulated by the hypothalamus [48]. A number of anorexigenic and orexigenic molecules are produced within or are secreted by the hypothalamus [20,44,17]. One of the anorectic molecules is the 82-amino acid neuropeptide nesfatin-1 (NEFA/nucleobindin2-encoded satiety and fat-influencing protein) [36]. Nesfatin-1 is cleaved from the N-terminus of a larger protein NEFA (also called nucleobindin2 or NUCB2) by prohormone convertases in neurons in several hypothalamic regions including the paraventricular (PVN), arcuate (ARC) and supraoptic nuclei, the lateral hypothalamic area and within brain stem neurons [20,12,16,14]. Nesfatin-1 possesses all of the anorectic activity of NUCB2.

Oh-I et al. have identified nesfatin-1 as a satiety molecule that likely activates the melanocortin pathway [36,32]. Intracerebroventricular (i.c.v.) injection of NUCB2 or nesfatin-1 inhibits food intake in a dose-dependent manner and results in total body fat and weight loss, whereas injection of an antibody specific to nesfatin-1 increases food intake in rats [36]. Nesfatin-1 suppresses both short and long term appetite,

**Abstract**

Nesfatin-1 has recently been identified as a hypothalamic and brain stem peptide that regulates feeding behavior. Here, we determined the ability of nesfatin-1 to cross the blood–brain barrier (BBB) of mice. We used multiple-regression analysis to determine that radioactively labeled nesfatin-1 injected intravenously entered the brain. The entry rate (Ki) of 131I-nesfatin-1 from blood-to-brain was 0.20 ± 0.02 µl/g min. This modest rate of entry was not inhibited by the administration of nonradioactive nesfatin-1, suggesting that BBB transport of nesfatin-1 into the brain is by a nonsaturable mechanism. High performance liquid chromatography (HPLC) and acid precipitation showed that most of the injected radiolabeled nesfatin-1 reached the brain as intact peptide, and capillary depletion with vascular washout revealed that 67% of 131I-nesfatin-1 crossed the BBB to reach the brain parenchyma. Efflux of labeled nesfatin-1 from brain back into blood was by way of bulk flow. These findings demonstrate that nesfatin-1 crosses the BBB in both the blood-to-brain and brain-to-blood directions by nonsaturable mechanisms.

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but other posttranslational products of NUCB2 (nesfatin-2 or nesfatin-3) have no effect on food intake. Nefat-in-1 acts independently of other known anorexigenic molecules, specifically leptin. For example, nesfatin-1 causes significant reductions in food intake when given i.c.v. to leptin-resistant obese Zucker rats and injection of antibodies to nesfatin-1 does not affect reductions in food intake caused by leptin injection. Thus, nesfatin-1 does not act through the leptin pathway but likely acts through the melanocortin system in the hypothalamus [36,18]. This suggests that nesfatin-1 might be functional in leptin resistance states as found in diet-induced obesity in humans [46,23,42,34]. Therefore, nesfatin-1 might provide an interesting target for antiobesity drug design [45]. Transport of feeding-related peptides and regulatory proteins across the blood–brain barrier (BBB) is an important pathway but likely acts through the melanocortin system in the hypothalamus [36,18]. This suggests that nesfatin-1 might be functional in leptin resistance states as found in diet-induced obesity in humans [46,23,42,34]. Therefore, nesfatin-1 might provide an interesting target for antiobesity drug design [45].

2. Materials and methods

2.1. Radioactive labeling of nesfatin-1 with 131I

Human nesfatin-1 (1–82) (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) was radioactively labeled with 131I (Perkin-Elmer Life Sciences, Boston, MA, USA) by the lactoperoxidase method [13]. Briefly, 5 μg of nesfatin-1 was mixed with 30 μl of 0.4 M sodium acetate (pH 5.6), 10 μl of lactoperoxidase (10 μg/ml), and 2 mCi of 131I. The reaction was started by adding 0.02 ng of H2O2 in a volume of 10 μl. Ten minutes later, an additional 0.02 ng of H2O2 was added. At the end of this second 10 min period, the iodinated nesfatin-1 (131I-nesfatin) was purified by filtration on a Sephadex G-10 column. An additional 0.02 ng of H2O2 was added. At the end of this second 10 min period, the iodinated nesfatin-1 (131I-nesfatin) was purified by filtration on a Sephadex G-10 column.

2.2. Measurement of the blood-to-brain influx rate

Male CD-1 mice (6–8 weeks old) were anesthetized with an intraperitoneal injection (i.p.) of 0.2 ml of urethane (40%). The jugular vein and right carotid artery were exposed. Mice were given an injection into the jugular vein of 0.2 ml of lactated Ringer’s solution with 1% BSA (LR-BSA) containing 300,000 cpm of 131I-nesfatin-1. After a specified time ranging from 1 to 60 min after intravenous injection (i.v.) of 131I-nesfatin-1, blood was collected from the right carotid artery, and the whole brain was removed and weighed at 1–10 min after the jugular injection and processed and analyzed to yield Ki and Vl. Regression lines yielding those values were compared as described below.

2.3. Determination of rate of clearance of 131I-nesfatin-1 from the serum

To determine the rate of clearance of 131I-nesfatin-1 from the serum, results were expressed as the percent of the injected dose in each milliliter of serum (%Inj/ml) and these values were plotted against time (min). The %Inj/ml was determined by the equation:

\[
\%\text{Inj}/\text{ml} = \frac{100 \times \text{CPM/ml serum}}{\text{mean CPM/injection}}
\]

The percent of the injected dose entering each gram of brain tissue (%Inj/g) was calculated at each time from the equation:

\[
\%\text{Inj}/\text{g} = \frac{100 \left( \frac{\text{Am}}{\text{Cpt}} - V_l \right) \text{Cpt}}{\text{Inj}}
\]

where Am/Cpt represents the brain/serum ratio at time t and Inj is the dose of 131I-nesfatin-1 injected i.v. Subtracting Vi, here assumed to be 10 μg/l, from the brain/serum ratio corrects for 131I-nesfatin-1 in the vascular space of the brain. Thus, the quantities expressed more nearly represent only the 131I-nesfatin-1 that has entered the brain tissue. The values for %Inj/g were plotted against time (min).

2.4. Determination of octanol/buffer partition coefficient

Lipid solubility of nesfatin-1 was measured by adding 1 × 105 cpm of 131I-nesfatin-1 to duplicate tubes each containing 0.5 ml of 0.25 M chloride-free sodium phosphate buffer (pH 7.5) and 0.5 ml of octanol. This solution was vigorously mixed for 1 min and then centrifuged at 5400 × g for 10 min to separate the two phases. Aliquots of 100 μl were taken in triplicate from each phase and total radioactivity counted in a gamma counter. The mean partition coefficient was expressed as:

\[
\text{Octanol/buffer partition coefficient} = \frac{\text{octanol phase cpm}}{\text{phosphate buffer phase cpm}}
\]

where Am is cpm/g of brain, Cpt(t) is cpm/μl of serum at time t and exposure time (Expt, in min) is measured by the term \( \left[ \frac{1}{2} \text{Cpt} \right] \). The linear portion of the relation between Am/Cpt ratios versus Expt was used to calculate K, and Vl. K, is reported with its error term.

To determine whether brain uptake of 131I-nesfatin-1 was saturable, 1 μg/mouse of nonradioactive nesfatin-1 was included in the i.v. injection of some mice. Blood was collected from the right carotid artery, and the whole brain was removed and weighed at 1–10 min after the jugular injection and processed and analyzed to yield Ki and Vl. Regression lines yielding those values were compared as described below.
2.5. Capillary depletion

The capillary depletion method [50] as modified for mice [22] was performed to determine whether the $^{131}$I-nesfatin-1 completely crossed the capillary wall of the BBB to enter the brain parenchyma. Anesthetized mice received an i.v. injection of 0.2 ml of LR-BSA and 300,000 cpm of $^{131}$I-nesfatin-1. To remove $^{131}$I-nesfatin-1 in the vascular space or loosely adhering to the luminal side of capillaries of the brain microvasculature, the vascular space was washed out as follows. Ten minutes after the i.v. injection into the jugular vein, the abdomen was opened and arterial blood was collected from the abdominal aorta. The thorax was then opened to expose the heart. The descending aorta was clamped, both jugular veins severed and 20 ml of LR perfused over 1 min into the left ventricle of the heart. After that, the mouse was decapitated and the whole brain removed, weighed and placed in an ice-cold glass homogenizer. The brain was homogenized (10 strokes) in 0.8 ml of physiological buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl$_2$, 1 mM MgSO$_4$, 1 mM Na$_2$HPO$_4$ and 10 mM D-glucose, pH 7.4). Dextran solution (1.6 ml of a 26% solution) was added to the homogenate, mixed and homogenized a second time (3 strokes). The homogenate was centrifuged at 5400 g for 15 min at 4 °C. The resulting supernatant (brain parenchymal fraction) and pellet (capillary fraction) were separated and the level of radioactivity for each fraction was determined using a gamma counter.

The parenchyma/serum and capillary/serum ratios ($\mu$l/g) were calculated by the equation:

$$\text{Ratio} = \frac{\text{cpm/g of tissue}}{\text{cpm/\mu l of serum}}$$

2.6. Stability of $^{131}$I-nesfatin-1 in serum and brain: acid precipitation

Male CD-1 mice (6–8 weeks old) were anesthetized with an i.p. injection of 0.2 ml urethane (40%). The left jugular vein and right carotid artery were then exposed. The mice were given an injection into the left jugular vein of 0.2 ml of LR-BSA containing 500,000 cpm of $^{131}$I-nesfatin-1 and 10, 30 or 60 min later brain and arterial blood was collected. The whole blood was centrifuged at 5400 × g for 10 min at 4 °C. The resulting supernatant (brain parenchymal fraction) and pellet (capillary fraction) were separated and the levels of radioactivity for each fraction were determined using a gamma counter. The resulting serum (50 µl) was added to 500 µl of 1% BSA in 0.25 M sodium phosphate buffer and then precipitated with 500 µl of 30% trichloroacetic acid. It was centrifuged at 5400 × g for 10 min at 4 °C and the resulting supernatant and pellet were counted. The whole brain was homogenized in 3 ml of LR-BSA for 10 strokes and 2 ml of brain homogenate was centrifuged at 5400 × g for 10 min at 4 °C. Brain supernatant (500 µl) was transferred to a new tube and 500 µl of 30% trichloroacetic acid added. This solution was centrifuged at 5400 × g for 10 min and the supernatant and the pellet separated. The levels of radioactivity in acidified serum and brain supernatants and pellets were counted in a gamma counter for 3 min. To determine degradation of $^{131}$I-nesfatin-1 that occurred after 40 min, the samples used to obtain carotid blood, and the samples were processed as described above. The percentage of radioactivity precipitated by acid in serum and brain was calculated by the following formula:

$$\%\text{ precipitated} = \frac{\text{pellet cpm}}{\text{pellet cpm} + \text{supernatant cpm}} \times 100$$

Correction for ex vivo degradation was made by dividing the values for the biological samples by the values for processing controls.

2.7. Stability of $^{131}$I-nesfatin-1 in serum and brain: HPLC

High-performance liquid chromatography (HPLC) was performed to determine whether the radioactivity in mouse brain and serum samples represented intact $^{131}$I-nesfatin-1. $^{131}$I-nesfatin-1 (10 × 10$^6$ cpm) was injected into the left jugular vein of each mouse (n = 2) and 10 min later blood was collected from the right carotid artery and the whole brain was removed. The whole blood was centrifuged at 5400 × g for 10 min at 4 °C. The resulting supernatant was lyophilized. The whole brain was collected in 3 ml of LR-BSA on ice and homogenized for 12 strokes. The brain homogenate was centrifuged at 5400 × g for 10 min at 4 °C and the resulting supernatant was lyophilized. The brain and serum processing control samples were used to determine any degradation that occurred during the processing. For the serum processing control, about 75,000 cpm were added to the bottom of a tube before the collection of arterial blood from a mouse. For the brain processing control, about 75,000 cpm were placed on the surface of a nonradioactive brain. The blood and brain samples for processing controls were then processed as described above. The lyophilized brain and serum samples were reconstituted in HPLC grade water. For the brain samples, the supernatant was again centrifuged at 20,000 × g for 20 min at 4 °C and then analyzed by reverse-phase HPLC on a C$_{18}$ column (Phenomenex, Onyx, Monolithic). Brain and serum samples (0.1 ml) were injected onto the column for the analysis and eluted with a gradient that linearly progressed from 100% of solution A (0.1% TFA in water) to 100% of solution B (0.1% TFA in acetonitrile) in 50 min. Fractions of the eluent were collected at 1 ml/min and counted on the gamma counter. Results were expressed as the percent per fraction of total cpm eluted.

2.8. Brain perfusion studies

Male CD-1 mice (6–8 weeks old) were anesthetized with an i.p. injection of 0.2 ml urethane (40%). $^{131}$I-nesfatin-1 was diluted in Zlokovic’s buffer (pH 7.4; 7.19 g/l NaCl, 0.3 g/l KCl, 0.28 g/l CaCl$_2$, 2.1 g/l NaHCO$_3$, 0.16 g/l KH$_2$PO$_4$, 0.17 g/l anhydrous MgCl$_2$, 0.99 g/l D-glucose and 10 g/l BSA added on the day of perfusion). The thorax was then opened, the heart was exposed and the descending thoracic aorta was clamped. Both jugular veins were severed. A 26-gauge butterfly needle was inserted into the left ventricle of the heart, and the buffer containing $^{131}$I-nesfatin-1 (100,000 cpm/ml) was infused at a rate of 2 ml/min for 1–5 min (n = 3). A 10 µl sample of the buffer solution was taken from the catheter tip to determine the exact counts per minute perfused. After perfusion, the butterfly needle was
removed, the mouse was decapitated and the brain removed. The brain was weighed and counted in a gamma counter for 3 min. The brain/perfusate ratio (µl/g) was calculated by the following formula:

\[
\frac{\text{Brain/perfusate ratio}}{\text{cpm/g of brain}} = \frac{\text{cpm/µl of perfusate}}{\text{g of brain}}
\]

2.9. Intracerebroventricular (i.c.v.) injection for determination of brain-to-blood efflux rate

A method previously described to quantify the rate of transport from brain-to-blood was used [2]. Mice were anesthetized with i.p. urethane. For each mouse, after the scalp was removed, a hole was made into the lateral ventricle of the brain (1.0 mm lateral and 0.5 mm posterior to the bregma) with a 26-gauge needle. Tubing covered all but the terminal 3.0–3.5 mm of the needle. The mice were given an injection of 1.0 µl of LR-BSA containing 10,000 cpm of ¹³¹I-nesfatin-1 into the lateral ventricle of the brain with 1.0 µl Hamilton syringe. The amount of radioactivity in the brain at t = 0 min were determined in mice that were overdosed with urethane before i.c.v. injection and decapitated 10 min later. The whole brain was removed at 0, 2, 5, 10 and 20 min after the i.c.v. injection (n = 3 for each time point). The levels of radioactivity for whole brain were counted in a gamma counter and the mean of the three mice at each time interval was used in subsequent calculations. The experiment was repeated so that two determinations of three mice each for each of the five time points were calculated. The log of the mean residual radioactivity per whole brain was plotted against time (min) to calculate the half-time disappearance rate. To test for self-inhibition of transport in other mice, nonradioactive nesfatin-1 (1 µg/mouse) was included in the injection and the brain was removed 10 min later. Results were expressed as the mean percent of radioactivity injected i.c.v. taken up per gram of whole brain (%Inj/brain).

2.10. Brain regions

Male CD-1 mice (6–8 weeks old) were anesthetized with an i.p. injection of 0.2 ml of urethane (40%). The jugular vein and right carotid artery were exposed. A total of 0.2 ml of LR-BSA containing 800,000 cpm of ¹³¹I-nesfatin-1 was injected into the left jugular vein. Blood was collected from the right carotid artery, and the brain was removed and dissected on ice into 11 regions (frontal, parietal and occipital cortices, hippocampus, hypothalamus, thalamus, striatum, mid-brain, pons-medulla, cerebellum and olfactory bulb). Each region was weighed before its level of radioactivity was determined in a gamma counter. The whole blood was centrifuged at 5400 x g for 10 min at 4 °C. The level of radioactivity was measured in serum (50 µl) and each brain region by counting in a gamma counter. Values for whole brain were calculated by adding the regional levels of radioactivity and regional weights. The brain/serum ratios (µl/g) for all regions and for whole brain were calculated. (Brain region/serum ratios were calculated as CPM/g of brain region/CPM/µl of serum.

2.11. Statistics

Statistical analysis was performed with the use of the Prism 4.0 program (GraphPad Software Inc., San Diego, CA, USA). Regression lines were calculated by the least-squares method and are reported with their correlation coefficient, r, n and p values. Regression lines were compared statistically with the Prism 4.0 program. Means are reported with their standard error terms and n. Two means were compared by t-test analysis.

3. Results

3.1. Clearance from the serum

Fig. 1 shows the clearance of ¹³¹I-nesfatin-1 from the serum for the first 60 min after injection into the jugular vein. The early phase of clearance from the serum for ¹³¹I-nesfatin-1 followed first-order kinetics. The inset shows the relation between the log of levels of radioactivity in arterial serum expressed as the %Inj/ml versus time after i.v. injection. Linear regression analysis showed a statistically significant relation between log(%Inj/ml) and time (t) (r = 0.71, p < 0.05, n = 10), for the first 10 min after i.v. injection (Fig. 1 inset). The half-time disappearance rate from serum was calculated from the inverse of the slope of this relation to be 9.46 min.

The percentage of the i.v. injected dose of ¹³¹I-nesfatin-1 taken up per gram of brain for the first 10 min after i.v. injection was 0.033 ± 0.013 %Inj/g as determined from the equation given in Section 2.

3.2. Brain uptake (influx) of ¹³¹I-nesfatin-1 from the blood into the brain

We measured the rate of unidirectional influx (Kt) from blood-to-brain for ¹³¹I-nesfatin-1. Fig. 2A shows the relation between
3.3. Capillary depletion study with washout

To determine whether $^{131}$I-nesfatin-1 was sequestered by brain endothelial cells or entered brain parenchyma, capillary depletion was performed with washout of the vascular space 10 min after i.v. injection. The brain parenchyma/saline radioactivity ($\mu$g/mouse) was measured to be $0.05 \pm 0.04 \mu$g/mouse ($n = 6$ mice) and was significantly higher than the capillary/saline ratio of $0.51 \pm 0.25 \mu$g/mouse ($n = 6$ mice, $p < 0.0311$) (Fig. 4). These results show that $^{131}$I-nesfatin-1 crossed the brain endothelial barrier of the cerebral cortex to enter the parenchymal space of the brain.

3.4. Brain perfusion of $^{131}$I-nesfatin-1

$^{131}$I-nesfatin-1 was found to cross the BBB with a $K_i$ of $0.3049 \pm 0.3847 \mu$g/l (n = 3/time, $p = 0.44$) when assessed by brain perfusion (Fig. 5).

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**Fig. 2** — (A) Multiple-time regression analysis of $^{131}$I-nesfatin-1 transport across the BBB. The unidirectional influx rate, $K_i$ (slope) was measured to be $0.20 \pm 0.02 \mu$g/min. $V_i$ (intercept) = $9.79 \pm 0.84 \mu$g/l, $r = 0.886$, $p < 0.0001$ and $n = 13$ mice. The brain/serum ratio of $^{131}$I-nesfatin first 20 min after i.v. injection is shown in the inset. (B) log(Serum cpm) of $^{131}$I-nesfatin at 1–10 min after i.v. injection. (C) The $K_i$ for $^{131}$I-nesfatin-1 (radioactive only) $0.23 \pm 0.06 \mu$g/min and $^{131}$I-nesfatin-1 + nonradioactive nesfatin-1 (+nonradioactive) $0.25 \pm 0.09 \mu$g/min 1–10 min after i.v. injections ($n = 10$ mice/group). There was no statistically significant difference between the two groups.

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brain at time zero ($V_i = 9.79 \pm 0.84 \mu$g/l), is expressed in units of $\mu$g of serum/l of brain tissue and approximates the vascular space in the brain. The inset shows the influx of $^{131}$I-nesfatin-1 was linear within the first 10 min, and the constant was $0.21 \pm 0.07 \mu$g/min ($V_i = 9.28 \pm 0.66 \mu$g/l, $r = 0.78$, $p < 0.02$, $n = 8$).

To determine the mechanism of brain uptake, we injected $^{131}$I-nesfatin-1 with or without 1 $\mu$g/mouse of nonradioactive nesfatin-1. In these experiments, the $K_i$ for $^{131}$I-nesfatin-1 (radioactive only) was $0.23 \pm 0.06 \mu$g/min and for $^{131}$I-nesfatin-1 + nonradioactive nesfatin-1 (+nonradioactive) was $0.25 \pm 0.09 \mu$g/min 1–10 min after i.v. injections ($n = 10$ mice/group). There was no statistically significant difference between the two groups (Fig. 2C). Fig. 2B shows log(Serum cpm) of $^{131}$I-nesfatin-1 at 1–10 min after i.v. injection. The half-time disappearance rate was $5.35$ min for $^{131}$I-nesfatin-1 only group and $8.98$ min for +nonradioactive group. This result indicates that $^{131}$I-nesfatin-1 crossed the BBB by a nonsaturable mechanism.

To determine the in vivo stability of $^{131}$I-nesfatin-1 in serum and brain, the two separate methods of acid precipitation and HPLC analysis was used. Acid precipitation of radioactivity recovered from serum averaged 92% intact at 10 min, 81% intact at 30 min and 78% at 60 min. In brain, 91% was intact at 10 min, 67% at 30 min and about 59% at 60 min. To further evaluate degradation, we analyzed the radioactivity extracted from brain and serum by HPLC 10 min after i.v. administration of $^{131}$I-nesfatin-1. For standard 15% eluted as free iodine and the rest (56%) as $^{131}$I-nesfatin-1 (Fig. 3A). Thirty-eight percent eluted as intact $^{131}$I-nesfatin-1 and 29% eluted as free iodine for a brain processing control (Fig. 3B). Twenty-seven percent of the serum processing control eluted in the same position as $^{131}$I-nesfatin-1 and the rest (46%) eluted as free iodine. After 10 min of i.v. circulation of $^{131}$I-nesfatin-1, 49% eluted as intact $^{131}$I-nesfatin-1 and 21% eluted as free iodine for brain sample (Fig. 3C) and 17% eluted as intact peptide and 42% as free iodine for serum sample. When we corrected for degradation during processing, 60% of total radioactivity from serum and 100% of the total radioactivity from brain eluted as intact $^{131}$I-nesfatin-1 10 min after i.v. injection.
3.5. Octanol/buffer partition coefficient

The mean octanol/buffer partition coefficient calculated to be 0.0085 ± 0.0012, n = 6. This gave a log value of −2.07, showing that \(^{131}\)I-nesfatin-1 is very hydrophilic.

3.6. Brain-to-blood transport (efflux) of \(^{131}\)I-nesfatin-1

Brain-to-blood efflux of \(^{131}\)I-nesfatin-1 was measured after i.c.v. injection. Fig. 6A shows the relation between log(brain cpm) and time was statistically significant, \(r = 0.76, n = 10, p < 0.0102\), demonstrating efflux from brain. The half-time disappearance rate calculated from the slope of this relation was 28.6 min. The %Inj/brain for \(^{131}\)I-nesfatin-1 and of \(^{131}\)I-nesfatin-1 + nonradioactive nesfatin-1 was calculated 10 min after i.c.v. injection (\(n = 10\) mice/group). The addition of nonradioactive nesfatin-1 did not inhibit \(^{131}\)I-nesfatin-1 efflux, \(^{131}\)I-nesfatin-1 [35.95 ± 9.41\% (\(^{131}\)I-nesfatin-1); 31.40 ± 7.76\% (\(^{131}\)I-nesfatin-1 + nonradioactive nesfatin)], suggesting that brain-to-blood efflux occurs by the nonsaturable mechanism of CSF reabsorption, termed bulk flow (Fig. 6B).

3.7. Brain regions

\(^{131}\)I-nesfatin-1 entered all regions of the brain. The olfactory bulb had the highest brain/serum ratio and was statistically different (\(p < 0.001\)) from whole brain (Fig. 7 and Table 1).

4. Discussion

Nesfatin-1 is a newly discovered satiety molecule detectable in neurons of the hypothalamus and nucleus of solitary tract...
(NTS) that mediates the anorexic action of NUCB2 in the CNS [36]. This neuropeptide has been added to an extensive list of feeding related-peptides, including leptin, ghrelin, orexins, insulin, agouti related peptide, adiponectin and cocaine-amphetamine regulated transcript peptide that have been identified in the last several years [3,30,31,37,40,43]. The BBB plays a dynamic role in regulating the passage of feeding-related peptides and regulatory proteins between the CNS and peripheral tissues [11]. In the present study, we found that radioactively labeled nesfatin-1 crosses the BBB in both the blood-to-brain and brain-to-blood directions by a nonsaturable system.

We determined whether radioactively labeled nesfatin-1 could cross the BBB in the blood-to-brain direction by using multiple-time regression analysis, a method that is highly sensitive and widely used to study of the penetration of peptides and proteins across the BBB [29,38,13]. The blood-to-brain unidirectional influx of $^{131}$I-nesfatin-1 was linear during the study period and the influx ($K_i$) rate was $0.20 \pm 0.02 \mu l/g min$, exhibiting a moderate rate of uptake for the peptide. This rate suggests a nonsaturable penetration of the BBB which is dependent on molecular size and lipophilicity. This is consistent with the low lipophilicity of nesfatin-1 as shown by its low octanol/buffer coefficient of 0.0085. Nonsaturable transport was confirmed by the uptake not being inhibited when we coinjected 1 mg/mouse of nonradioactive nesfatin-1. It is possible that a higher dose might have unmasked saturable transport, but this is unlikely because of low influx rate nesfatin-1. Therefore, we think that $^{131}$I-nesfatin-1 penetrates the brain through the nonsaturable mechanism of transmembrane diffusion. Several feeding-related peptides,
including melanin-concentrating hormone, CART, orexin, NPY and PYY1-36 are able to cross the BBB via transmembrane diffusion [24-28,33,35,44]. Such transport has usually been related to lipid solubility [9], hydrogen bonding [15], and other factors such as charge, protein binding or tertiary structure [41]. It has been shown that substances with molecular weight more than 5000 Da can have BBB transport into the brain by transmembrane diffusion [9]. (Molecular weight of nesfatin-1 is 9552.)

The percent of $^{131}$I-nesfatin-1 entering each gram of brain in the first 10 min after i.v. injection was 0.033 ± 0.013. This amount of nesfatin-1 entering the brain may be enough to produce therapeutic effect. Several substances that are known to exert central effects after peripheral administration have similar values; for example, morphine (0.02 %Inj/g), domoic acid (0.002 %Inj/g) and PACAP (0.11 %Inj/g) [7,39,5]. The percent of the i.v. injected dose taken up per ml of brain is important as a function of the rate of entry. However, the short half-time disappearance rate from serum of 9.46 min will likely limit the therapeutic use of native nesfatin-1.

To characterize the in vivo stability of $^{131}$I-nesfatin-1 in brain and serum after i.v. injection, we used acid precipitation and HPLC analysis. Both acid precipitation and HPLC, demonstrated that the radioactivity entering the brain from the blood mostly represents intact $^{131}$I-nesfatin-1.

The distribution of $^{131}$I-nesfatin-1 among capillary and parenchymal compartments of the brain was determined by capillary depletion 10 min after i.v. injection of $^{131}$I-nesfatin-1. We showed that most of $^{131}$I-nesfatin-1 was found in the brain parenchyma (67% of the total radioactivity), rather then being sequestered by capillaries.

The brain perfusion method with a blood-free perfusion buffer eliminates the influence on transport of factors such as binding to circulating proteins degradation in blood or sequestration by peripheral tissues [49,47].

We next demonstrated that $^{131}$I-nesfatin-1 also crossed the BBB in the brain-to-blood direction by a nonsaturable mechanism. Brain-to-blood efflux of $^{131}$I-nesfatin-1 was measured after i.c.v. injection and the relation between log(brain cpm) and time was statistically significant after the injection of $^{131}$I-nesfatin-1. The half-time disappearance rate calculated from the slope of this relation was 28.6 min. This rate indicates that brain-to-blood passage likely occurs with the reabsorption of cerebrospinal fluid back into the circulating blood, a mechanism termed bulk flow. If nesfatin-1 crosses the BBB via a saturable brain-to-blood transport system, the half-time efflux rate would be significantly shorter [6]. The addition of unlaabeled nesfatin-1 did not inhibit $^{131}$I-nesfatin-1 efflux, supporting that brain-to-blood efflux occurs by a nonsaturable mechanism.

We compared the regional distribution within the brain of i.v.-injected, radioactively labeled nesfatin-1. We measured the brain/serum ratios of $^{131}$I-nesfatin-1 for each brain regions after i.v. injection. $^{131}$I-nesfatin-1 entered all regions of the brain, but the olfactory bulb had the higher brain/serum ratios and was statistically different from whole brain. Except for the olfactory bulb, the rest of the brain regions were not statistically different from whole brain. Regional uptakes by brain of some peptides have been shown to correlate with the distribution of their receptors in the CNS [3,4]. However, a unique nesfatin-1 receptor has yet to be identified and thus the actual site of action of the peptide to inhibit food intake has not been determined. Recently, a study reported that NUCB2 protein is identified in the 3T3-L1 adipocyte proteome [1]. It suggests that nesfatin-1 may be produced in the periphery. If nesfatin-1 circulates in blood, then its ability to cross the BBB may mean that peripheral sources can affect brain activity.

In conclusion, the results from the present study demonstrate that nesfatin-1 can cross the BBB of mice in both the blood-to-brain and brain-to-blood directions by nonsaturable mechanisms. Several hypothalamic peptides like nesfatin-1 contributing to leptin-independent melanocortin signaling are potential targets for the development of pharmacological treatments for obesity. Those peptides have been demonstrated to exert multiple actions in brain in addition to their effects on food intake, including effects on autonomic activity, neuroendocrine function and locomotor behaviors. It is likely that nesfatin-1 will be observed to exert multiple CNS actions as well.

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