Characterization of Blood-Brain Barrier Permeability to PYY$_{3–36}$ in the Mouse

NAOKO NONAKA, SEIJI SHIODA, MICHAEL L. NIEHOFF, and WILLIAM A. BANKS

Oral Anatomy, School of Dentistry, Showa University, Tokyo, Japan (N.N.); First Anatomy, School of Medicine, Showa University, Tokyo, Japan (N.N., S.S.); Geriatric Research, Education, and Clinical Center, Veterans Affairs Medical Center-St. Louis and Division of Geriatrics, Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri (N.N., M.L.N., W.A.B.)

Received March 18, 2003; accepted May 7, 2003

ABSTRACT

Peptide YY$_{3–36}$ (PYY) has emerged as an important signal in the gut-brain axis, with peripherally administered PYY affecting feeding and brain function. For these effects to be direct, PYY would have to cross the blood-brain barrier (BBB). Here, we determined the permeability of the BBB to PYY radioactively labeled with $^{131}$I (I-PYY). Multiple-time regression analysis showed the unidirectional influx rate ($K_i$) from blood-to-brain for I-PYY to be 0.49 ± 0.19 μl/g-min, a rate similar to that previously measured for leptin. Influx was not inhibited by 1 μg/mouse of unlabeled PYY, suggesting PYY crosses the BBB by transmembrane diffusion. About 0.176% of the i.v.-injected dose of I-PYY was taken up by brain, an amount similar to that for other peptides important in gut-brain communication. Capillary depletion showed that 69% of I-PYY crossed the BBB to enter the parenchymal space of the brain, and high-performance liquid chromatography demonstrated that the radioactivity in this space represented intact I-PYY. After intracerebroventricular injection, I-PYY crossed from brain to blood by the mechanism of bulk flow. We conclude that PYY crosses in both the blood-to-brain and brain-to-blood directions by nonsaturable mechanisms. Passage across the BBB provides a mechanism by which blood-borne PYY can affect appetite and brain function.

Peptide YY has emerged as a major component of the gut-brain axis regulation of feeding, body weight, and nutritional status. It is a member of the neuropeptide Y family that includes neuropeptide Y (NPY), pancreatic polypeptide, and two forms of peptide YY, a 36-amino acid form and the 3- to 36-amino acid form (PYY). Each of these forms acts through the NPY receptors (Larhammar, 1996). For example, PYY$_{1–33}$ is an agonist at the Y1/Y2 receptors and promotes feeding, whereas PYY$_{3–36}$ is an antagonist at the Y2 receptor and inhibits feeding (Grandt et al., 1994b). PYY is abundant in human blood (Grandt et al., 1994a) and is released from endocrine cells found throughout the small intestine in proportion to the caloric content of a meal (Ekblad and Sundler, 2002). Blood-borne PYY can affect appetite and influence neuronal activity at the arcuate nucleus (Batterham et al., 2002).

The arcuate nucleus in the adult is clearly separated from the peripheral circulation and median eminence by the endothelial and ependymal arms of the blood-brain barrier (BBB) (Rethelyi, 1984; Peruzzo et al., 2000). Therefore, PYY would have to negotiate the BBB to reach the receptors in the arcuate nucleus. The BBB controls the exchange of peptides and regulatory proteins between the central nervous system (CNS) and blood (Banks and Kastin, 1985, 1993, 1996; Kastin et al., 1990; Begley, 1992; Brownlee and Williams, 1993) and has emerged as a major regulator of communication between the central nervous system and the peripheral tissues (Banks and Kastin, 1990). One area where this regulation is particularly clear is in the area of feeding hormones. Leptin (Banks et al., 1996), ghrelin (Banks et al., 2002c), melanocyte-stimulating hormone (Wilson et al., 1984), cocaine- and amphetamine-regulated transcript (Kastin and Akerstrom, 1999a), and insulin (Baura et al., 1993, 1997; Banks and Kastin, 1998) have all been shown to cross the BBB by saturable and nonsaturable mechanisms to a substantial extent, as have cytokines, which can also influence feeding behavior (Banks et al., 1995a, 2001a). Other members of the NPY family, including pancreatic polypeptide (Yokel, 1983) and NPY (Kastin and Akerstrom, 1999d), have been shown to cross the BBB.

Here, we determined whether PYY$_{3–36}$ can cross the BBB.

ABBREVIATIONS: NPY, neuropeptide Y; unlabeled PYY, unlabeled peptide YY$_{3–36}$; BBB, blood-brain barrier; CNS, central nervous system; I-PYY, $^{131}$I-peptide YY$_{3–36}$; Tc-Alb, 99mTc-albumin; LR, lactated Ringer’s solution; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; %Inj/ml, percentage of the injected dose present in a milliliter of serum.
of the mouse. We investigated both blood-to-brain and brain-to-blood pathway using radioactively labeled PYY.

Materials and Methods

Radioactive Labeling of PYY and Albumin. PYY is a small peptide of 4049.71 mol. wt. with a tyrosine at the amino-terminal that is available for iodination. PYY purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA) was radioactively labeled with 131I (PerkinElmer Life Sciences, Boston, MA) by the lactoperoxidase method. Briefly, 5 μg of PYY was mixed with 30 μl of 0.4 M sodium acetate (pH 5.6), 10 μl of lactoperoxidase (10 μg/ml), and 2 μCi 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, radioactively labeled PYY (I-PYY) was purified on a Sephadex G-10 column. Albumin was labeled with 99mTc (Tc-Alb) by mixing with stannous tartrate, adjusting to pH 2.0 to 3.0 with 0.2 M HCl, and incubating for 20 min.

Measurement of the Unidirectional Influx Rate. Male ICR mice (2 months old) were anesthetized with an i.p. injection of 0.2 ml of urethane (40% solution). The left jugular vein and right carotid artery were then exposed and the mice given an injection into the left jugular vein of 0.2 ml of lactated Ringer’s solution (LR) containing 250,000 cpm of I-PYY. Blood was collected from the right carotid artery, and the whole brain was removed and weighed at 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 min after the i.v. injection. The whole blood was centrifuged at 5,400g for 10 min at 4°C and the level of radioactivity measured in 50 μl of the serum. The levels of radioactivity in serum and in whole brain were counted in a gamma counter for 3 min. The brain/serum ratios for whole brain were calculated and the unidirectional influx rate (i.i. units of microliters per gram-minute) and the initial volume of distribution in brain at time 0 (V0, in units of microliters per gram) was determined by multiple-time regression analysis with the following formula:

\[ \text{Am/Cpt} = K_i [f_i Cpt(r/d)/Cpt + V_i] \]

where Am is cpm per gram of brain, Cpt is cpm per microliter of serum, and exposure time (Expt) is measured by the term \([f_i Cpt(r/d)/Cpt]\). The linear portion of the relation between Am/Cpt ratios versus Expts was used to calculate \(K_i\) and \(V_i\). \(K_i\) is reported with its error term.

To test for saturation of blood to brain passage, other mice were given an injection into the left jugular vein of 0.2 ml of LR containing 250,000 cpm of I-PYY plus 1 μg/mouse of unlabeled PYY.

Capillary Depletion. Male ICR mice (2 months old) were anesthetized with an i.p. injection of 0.2 ml of urethane (40% solution). The left and right jugular veins were exposed. Mice received an i.v. injection of I-PYY and Tc-Alb. Five minutes after the i.v. injection, the abdomen was opened and arterial blood was collected from the abdominal aorta. The thorax was then opened, the descending aorta 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 by 10 vertical strokes in 0.8 ml of physiological buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl2, 1 mM MgSO4, 1 mM KH2PO4, and 10 mM Na-glucose, pH 7.4). Dextran solution (1.6 ml of a 2% solution) was added to the homogenate, mixed, and homogenized with an additional three vertical strokes. The homogenate was centrifuged at 5,400g for 15 min at 4°C. The resulting supernatant (brain parenchymal fraction) and pellet (capillary fraction) were separated.

Brain Perfusion of I-PYY and I-PYY + Unlabeled PYY. Male ICR mice (2 months old) were anesthetized with an i.p. injection of urethane 40% solution (0.2 ml). I-PYY was diluted in Zlokovic’s buffer (pH 7.4; 7.19 g NaCl, 0.3 g KCl, 0.28 g CaCl2, 2.1 g NaHCO3, 0.16 g KH2PO4, 0.17 g anhydrous MgCl2, 0.89 g d-glucose, and 10 g/l bovine serum albumin added on the day of perfusion). The heart was exposed by opening the thorax. The descending thoracic aorta was clamped and the right and left jugular veins severed. A 21-gauge butterfly needle was inserted into the left ventricle of the heart, and the buffer containing I-PYY (250,000 cpm/ml) was infused at a rate of 2 ml/min for 1, 2, 3, or 5 min. In other mice, unlabeled PYY (1 μl/ml) was included in the I-PYY. After perfusion, the butterfly needle was removed, the mouse was decapitated and the brain removed. The brain was weighed and counted in

\[ \%\text{Inj/g} = \frac{[\text{Am/Cpt} - \text{V}i]\%\text{Inj/ml}}{1000} \]

where Inj is the dose administered. The percentage of the injected dose taken up per gram of brain (%Inj/g) was calculated at each time with the following formula:

\[ \%\text{Inj}/\text{ml} = 100\text{[Cpt/Inj]} \]

Stability of I-PYY in Serum and Brain. Male ICR mice (2 months old) were anesthetized with an i.p. injection of 0.2 ml of urethane (40% solution). 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 containing 200,000 cpm of I-PYY. Blood was collected from the right carotid artery, and the whole brain was removed at 1, 5, and 10 min after i.v. injection. The whole blood was centrifuged at 5,000g for 10 min at 4°C. Of the resulting serum, 50 μl was added to 250 μl of 1% bovine serum albumin in 0.25 M sodium phosphate buffer, and then precipitated with 250 μl of 30% trichloroacetic acid. The whole brain was homogenized in 3 ml of LR for 10 s and 2 ml of brain homogenate was centrifuged at 5,400g for 30 min at 4°C. A volume of 250 μl of 30% trichloroacetic acid was added to 250 μl of the brain supernatant and mixed. The acidified solutions of serum and brain were centrifuged at 5,400g for 10 min at 4°C. The levels of radioactivity in the acidified serum and brain supernatants and pellets were counted in a gamma counter for 3 min. The percentage of precipitate radioactivity in serum and brain was calculated by the following formula:

\[ 100\%\text{(pellet cpm)}/(\text{pellet cpm} + \text{supernatant cpm}) \]
a gamma counter for 3 min. The brain/perfusate ratio (microliters per gram) was calculated by the following formula:

\[
\text{brain/perfusate ratio} = \frac{\text{cpm of brain}}{[\text{cpm/\mu l of serum}][\text{g of brain}]].
\]

**Intracerebroventricular (i.c.v.) Injection for Measurement of Brain-to-Blood Passage.** Male ICR mice (2 months old) were anesthetized with an i.p. injection of 0.2 ml of urethane (40% solution). For each mouse, the skin was removed from the skull and a hole 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 to 3.5 mm of the needle. The mice were given an injection of 1.0 \( \mu l \) of LR containing 10,000 cpm of I-PYY into the lateral ventricle of the brain. The amount of radioactivity in the brain at \( t = 0 \) was estimated in mice that had been overdosed with urethane. The whole brain was removed at 0, 2, 5, 10, and 20 min after the injection, and three mice were studied at each time interval. The levels of radioactivity for whole brain was counted in a gamma counter for 3 min, and the mean of the three mice at each time interval was used in subsequent calculations. The experiment was repeated so that two determinations for each of the five time points (each determination the mean of three mice) was calculated.

The log of the mean residual radioactivity per whole brain was plotted against time and the slope used to calculate the half-time disappearance rate. In other mice, unlabeled PYY (1 \( \mu g/mouse \)) was included in the injection and the brain removed 10 min after the i.c.v. injection. The level of radioactivity in whole brain was determined with a gamma counter and divided by the cpm injected to yield the percentage of injected i.c.v. that was retained by whole brain (%Inj/brain).

**Statistics.** Statistical analysis was performed with the use of the Prism 3.0 program (GraphPad Software, Inc., San Diego, CA). Regression lines were calculated by the least-squares method and are reported with their correlation coefficient, \( r \), \( n \), and \( p \) values. Means are reported with their standard error terms and \( n \). Two means were compared by \( t \) test analysis.

**Results**

We first measured the rate of unidirectional influx (\( K_i \)) from blood to brain for I-PYY. Figure 1 shows the relation between brain/serum ratios (microliters per gram) and exposure time (minutes). The \( K_i \) for I-PYY into brain was 0.49 ± 0.19 \( \mu l/g \cdot \min \), \( r = 0.682, n = 10, p < 0.05 \). We next determined whether the mechanism by which I-PYY crossed the BBB was saturable. We did this by injecting I-PYY with or without 1 \( \mu g/mouse \) of nonradioactive PYY. The brain/serum ratios of I-PYY (11.02 ± 0.16 \( \mu l/g \)) and of I-PYY + unlabeled PYY (12.4 ± 0.31 \( \mu l/g \)) 5 min after i.v. injection (\( n = 14 \) mice/group) were statistically significant (\( p < 0.05 \); Fig. 1, inset).

To determine whether I-PYY crossed the BBB to enter brain parenchyma, we performed capillary depletion. Results are reported for experiments that used vascular washout and after correction for Tc-Alb 5 min after i.v. injection. The brain parenchyma/serum ratio (microliters per gram) was measured to be 2.47 ± 0.07 \( \mu l/g \) (\( n = 4 \) mice) and was significantly higher than the capillary/serum ratio of 1.10 ± 0.18 \( \mu l/g \) (\( n = 5 \) mice, \( p = 0.0015 \)). These results show that the majority of material taken up by brain had crossed the BBB to enter the parenchyma by 5 min after injection.

We then determined the amount of the intravenously injected dose that entered the brain. To do this, we first calculated the percentage of the injected dose present in 1 ml of serum (%Inj/ml). Figure 2A shows the log (%Inj/ml) of I-PYY at various times after i.v. injection. A statistically significant relation existed between log (%Inj/ml) and time (\( r = 0.71, p < 0.001, n = 5 \) mice/time). The half-time disappearance rate from serum was calculated from the slope of this relation to be 13.1 min. Figure 2B shows the percentage of the injected dose of I-PYY found per gram of brain (%Inj/g). The peak value for %Inj/g was at about 2 min after i.v. injection and was measured to be 0.176 ± 0.035, \( r = 0.04, n = 5 \) mice/time.
dose taken up per gram of brain (\%Inj/g) 1 to 10 min after i.v. injection. The peak value for \%Inj/g was at about 2 min after i.v. injection and was 0.176 ± 0.035, n = 5 mice/time.

Table 1 shows results for acid precipitation at 1, 5, and 10 min after i.v. injection. The amount of radioactivity that precipitated with acid had decreased little during the 10-min study time. These results show that essentially all of the radioactivity in brain and serum remained attached to peptide during the course of the study.

I-PYY was found to cross the BBB at a rate of 2.34 ± 0.74 μl/g (n = 18) when assessed by brain perfusion (Fig. 3). This is a rate faster than after i.v. injection.

Figure 4 shows the relation between percentage of total cpm and HPLC fraction number for processing controls and for samples taken 5 min after i.v. injection. For the brain processing control, 42% eluted in the same position as I-PYY, and 69% of the serum processing control eluted as intact I-PYY. Of the radioactivity recovered 5 min after i.v. injection of I-PYY, 37% eluted as intact I-PYY for brain and 66% for serum (Fig. 4, bottom, show the mean of two experiments). Correcting for degradation during processing, we calculated that 88% of radioactivity from brain and 96% of radioactivity from serum eluted as intact I-PYY 5 min after i.v. injection.

Brain-to-blood efflux of I-PYY was measured after i.c.v. injection. Figure 5 shows the relation between log (brain cpm) and time was statistically significant, r = 0.65, n = 10 mice, p < 0.001, demonstrating efflux from brain. The half-time disappearance rate calculated from the slope of this relation was 37.8 min. The \%Inj/brain for I-PYY and of I-PYY time disappearance rate calculated from the slope of this efflux does not exit.

Discussion

PYY has joined a growing list of important peptides secreted by the gastrointestinal tract that influence feeding behavior by acting within the CNS. PYY is released in response to feeding in proportion to the calorie load of the meal, inhibiting further food intake. A limited, but diverse number of pathways is available by which a blood-borne substance can influence the CNS. Direct passage of a substance across the BBB is one of the them. Passage across the BBB has been described for several peptides and regulatory proteins with effects on feeding, including leptin (Banks et al., 1996, 2002b; Banks, 2001; Banks and Lebel, 2002), pituitary adenylate cyclase-activating polypeptide (Banks et al., 1993; Nonaka et al., 2002), tumor necrosis factor-α (Banks et al., 1995a, 2001b), interleukin-6 (Banks et al., 1994, 1995a, 2001a), and insulin (Baura et al., 1993; Banks et al., 1997, 1999, 2000; Banks and Kastin, 1998), ghrelin (Banks et al., 2002c), melanocyte-stimulating hormone (Wilson et al., 1984), and cocaine- and amphetamine-regulated transcript (Kastin and Akerstrom, 1999a). Here, we found that PYY crosses in both the blood-to-brain and brain-to-blood directions by nonsaturable mechanisms.

We first determined whether intravenous I-PYY could cross the BBB and measured its unidirectional influx rate (K). It’s K of 0.49 ± 0.19 μl/g-min is similar to that of similar sized peptides shown to cross the BBB by nonsaturable mechanisms (Banks et al., 1995b, 2002a,c; Kastin and Akerstrom, 1999a,b,c). To determine whether the mechanism of entry had a saturable component, we included 1 μg of nonradioactive PYY in the injection, an amount shown to substantially inhibit transport systems for peptides and regulatory proteins such as leptin (Banks et al., 1996, 2002b; Banks, 2001; Banks and Lebel, 2002), pituitary adenylate cyclase-activating polypeptide (Banks et al., 1993; Nonaka et al., 2002), tumor necrosis factor-α (Banks et al., 1995a, 2001b), interleukin-6 (Banks et al., 1994, 1995a, 2001a), and insulin (Baura et al., 1993; Banks et al., 1997, 1999, 2000; Banks and Kastin, 1998). Inclusion of unlabeled PYY did not inhibit uptake of I-PYY, showing that passage across the BBB was most likely by transmembrane diffusion. However, there was a small, statistically significant increase with inclusion of unlabeled PYY. Such a paradoxical increase is sometimes caused by saturable brain to blood transport or blood-borne binding factors. These possibilities were considered in subsequent experiments.

Capillary depletion studies confirmed that 69% of the total radioactivity taken up by brain after intravenous injection crossed the BBB to enter the parenchymal space of the brain. HPLC confirmed the stability of the labeled PYY in brain and blood. Both acid precipitation and HPLC demonstrated that the radioactivity entering the brain represented intact I-PYY.

The percentage of the intravenously injected dose taken up per milliliter of brain is a function of the rate of entry and presentation to the brain via the circulation. As shown in Fig. 2A, the half-time disappearance rate from blood was 13.1 min. The \%Inj/g is important in determining an effective intravenous dose and is a function of BBB permeability and blood concentration. As shown in Fig. 2B, the peak value for \%Inj/g occurred about 2 min after i.v. injection and was 0.176 ± 0.035% Inj/g. This is similar to the uptake of other
peptides and proteins, such as [Tyr$^{10}$]secretin-27 (Banks et al., 2002a) and ghrelin (Banks et al., 2002c), that are known to effect brain function by virtue of their abilities to cross the BBB. Secretin has been shown to have a peak $%\text{Inj/g}$ of 0.180, whereas human ghrelin was 0.063.

Brain perfusion was also used to examine whether I-PYY could cross the BBB. Results showed that I-PYY crossed the BBB by a nonsaturable mechanism and so agreed with the i.v. results. However, the entry rate was 4 to 5 times faster as measured by brain perfusion. One mechanism that might explain these results is the presence of a blood-borne binding factor. Because brain perfusion replaces blood with buffer, the effects of binding factors is negated. This could also explain the paradoxical increase seen when unlabeled PYY was injected intravenously.

We found that PYY could also cross from brain to blood. The relation between log (brain cpm) and time after the i.c.v. injection of I-PYY was statistically significant, and the half-time disappearance rate was 37.8 min. This rate and the finding that there was not a saturable component shows that brain-to-blood passage is likely to occur with the reabsorption of cerebrospinal fluid into the blood. Brain-to-blood passage either by saturable or nonsaturable mechanisms can contribute significantly to blood levels and affect the functioning of peripheral tissues (Clark et al., 1983; Yao et al., 1993; Banks and Kastin, 1997; Chen et al., 1997; Martins et al., 1997; Chen and Reichlin, 1998). The lack of a saturable component makes it unlikely that efflux contributes to the
paradoxical increase in I-PYY uptake when unlabeled PYY was co-injected intravenously.

In conclusion, we found that PYY crosses the BBB bidirectionally by nonsaturable processes. The amount of PYY entering the brain after CNS injection is an amount that has been found for similar sized substances to induce effects on the CNS. A brain-to-blood efflux of PYY suggests that PYY been found for similar sized substances to induce effects on the CNS. A brain-to-blood efflux of PYY mediates its effects on the CNS.

References


Banks WA and Kastin AJ (1998) Saturable transport of insulin from plasma into the central nervous system across the BBB may be an important mechanism by which blood-borne PYY mediates its effects on the CNS.