## Research Article

# *In Vivo* Transport of a Dynorphin-like Analgesic Peptide, E-2078, Through the Blood–Brain Barrier: An Application of Brain Microdialysis

Tetsuya Terasaki,[1] Yoshiharu Deguchi,[1] Hitoshi Sato,[1] Ken-ichi Hirai,[1] and Akira Tsuji[1,2]

*Received October 18, 1990; accepted February 3, 1991*

*In vivo* transport through the blood–brain barrier (BBB) has been demonstrated for a dynorphin-like analgesic peptide, $CH_3$-[$^{125}$I]Tyr-Gly-Gly-Phe-Leu-Arg-$CH_3$Arg-D-Leu-$NHC_2H_5$ ([$^{125}$I]E-2078). A remarkable time-dependent increase in the distribution volume of [$^{125}$I]E-2078 in the brain parenchyma separated from blood vessels and capillaries was observed during a brain perfusion. The distribution volume of [$^{125}$I]E-2078 in the brain parenchyma after 20 min of perfusion was $2.18 \pm 0.09$ µl/g brain (mean ± SE) and was significantly greater than the distribution volume of [$^3$H]inulin ($0.994 \pm 0.138$ µl/g brain), providing *in vivo* evidence for the penetration of [$^{125}$I]E-2078 into the brain parenchyma. Brain microdialysis was carried out to collect directly the brain interstitial fluid (ISF) during the brain perfusion of [$^{125}$I]E-2078. No metabolite of [$^{125}$I]E-2078 in the brain ISF was found by high-performance liquid chromatographic analysis of the brain dialysate. The concentrations of [$^{125}$I]E-2078 and [$^{14}$C]sucrose in the brain ISF were estimated based on an *in vitro* evaluation of dialysis clearance. The concentration ratio of [$^{125}$I]E-2078 between the brain ISF and the brain perfusate was determined to be $2.92 \times 10^{-1} \pm 0.50 \times 10^{-1}$ and was approximately 100 times higher than that of [$^{14}$C]sucrose ($2.71 \times 10^{-3} \pm 1.43 \times 10^{-3}$), demonstrating transport of [$^{125}$I]E-2078 through the BBB *in vivo*. On the other hand, no remarkable difference in the cerebrospinal fluid (CSF)-to-perfusate concentration ratios of [$^{125}$I]E-2078 and [$^{14}$C]sucrose was observed, indicating little contribution of the blood–CSF barrier (BCSF barrier) transport to the penetration of [$^{125}$I]E-2078 into the brain.

KEY WORDS: brain microdialysis; dynorphin-like analgesic peptide; basic peptide; blood–brain barrier (BBB) transport; brain perfusion; brain interstitial fluid; blood–cerebrospinal fluid (BCSF) barrier; absorptive-mediated endocytosis.

## INTRODUCTION

For the delivery of pharmacologically active peptides into the brain, transport through the blood–brain barrier (BBB), which is formed by the endothelial cells of brain capillaries, is the most essential property. Recently, several studies have focused on peptide transport using *in vitro* isolated brain capillaries (1–3). To examine quantitatively the peptide penetration into the brain under physiological conditions, *in vivo* transport experiments (4–8) would also provide valuable insights. Especially, the brain microdialysis, which has been developed to measure neurotransmitters in the brain interstitial fluid (5,7), and the coupling of the brain perfusion and the capillary depletion technique (8,9) are useful to obtain direct evidence for *in vivo* transport through the BBB.

A synthetic octapeptide, E-2078 (Fig. 1), was developed to overcome instability of the native opioid peptide, dynorphin-(1-8), against peptidases (10). The analgesic effect of E-2078 has been reported to be severalfold greater than that of morphine after systemic administration in mice (11). Therefore, E-2078 is considered a potential analgesic drug and an appropriate model compound for the study of peptide penetration into the brain. We have previously shown that E-2078 is transported into isolated bovine brain capillary *in vitro* by an absorptive-mediated endocytosis (3), but direct evidence for the BBB transport of the peptide under physiological conditions has not been provided yet. Therefore, the purpose of the present study is to test whether E-2078 is transported through the BBB *in vivo* and the BBB is the major site at which the peptide is delivered into the brain.

## MATERIALS AND METHODS

### Animals

Male Wistar rats weighing 200–250 g were purchased from Sankyo Laboratory Co. (Toyama, Japan). They had free access to food and water.

### Chemicals

A dynorphin-like analgesic peptide, [*N*-methyl Tyr$^1$, *N*-methyl Arg$^7$, D-Leu$^8$]dynorphin-A-(1-8)ethylamide (E-2078), and its iodinated compound, [$^{125}$I]E-2078, with a specific

[1] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan.

[2] To whom correspondence should be addressed.



0724-8741/91/0700-0815$06.50/0 © 1991 Plenum Publishing Corporation

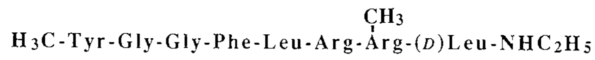


**Fig. 1.** Structure of a dynorphin-like analgesic peptide, E-2078.

activity of 0.82–21.4 mCi/mg, were kindly supplied from Eisai Co., Ltd. (Tokyo). [$^3$H(G)]Inulin (473 μCi/mg), [$^{14}$C(U)]sucrose (5.0 mCi/mmol), and Protosol (tissue solubilizer) were purchased from New England Nuclear Corp. (Boston, MA). Clear-sol (xylene-based liquid scintillation cocktail) was purchased from Nacalai Tesque Inc. (Kyoto, Japan), and Ketaral 50 (ketamine hydrochloride) from Sankyo Co., Ltd. (Tokyo). Dextran (industrial grade, MW 71,500), bovine serum albumin (BSA; Fraction V), and xylazine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals were of reagent grade and were used without further purification.

### *In Situ* Brain Perfusion Study

*In vivo* transport experiments were carried out with the combination of the brain perfusion method and the capillary depletion technique (8,9) or brain microdialysis (5,7,12). Rats were anesthetized with intramuscular doses of ketamine hydrochloride (235 mg/kg) and xylazine (2.3 mg/kg). The right external carotid artery was catheterized for the infusion to internal carotid artery with polyethylene tubing (SP-10) filled with sodium heparin (100 IU/ml). In this study, the principal tributaries of the carotid artery such as pterygopalatine, occipital, and superior arteries were not cauterized. The left common carotid artery was ligated in order to prevent influx from the systemic circulation of the peptide. Then the perfusion buffer [141 m*M* NaCl, 4.0 m*M* KCl, 2.8 m*M* $CaCl_2$, 1.0 m*M* $MgSO_4$, 10 m*M* *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (Hepes), 10 m*M* D-glucose, and 0.1% BSA, pH 7.4] containing 1 μCi/ml of [$^{125}$I]E-2078 or 2 μCi/ml of [$^3$H]inulin, oxygenated for 3 min with 95% $O_2$–5% $CO_2$, was perfused from the catheterized tubing at the rate of 1.0 ml/min, which is the blood flow rate of the brain in conscious rats (1.64 ± 0.11 ml/min/g brain) (13) multiplied by half the brain weight, 0.638 ± 0.012 g (12). As soon as the perfusion started, the right common carotid artery was ligated to avoid mixing with the blood from the systemic circulation. After a designated interval, the perfusion buffer without a substrate was infused through the same catheter at the same flow rate for 2 min to wash out the substrate in the vascular space. Then the perfusion was terminated by decapitation, and the ipsilateral hemisphere at the injected side was dissected. When [$^3$H]inulin was perfused, the perfused brain was solubilized in 3 ml of Protosol at 55°C for 4 hr in a glass vial, and 0.6 ml of 30% hydrogen peroxide was added to the vial. The sample was kept standing at room temperature for 15 min, incubated at 55°C for 30 min, and neutralized with 128 μl of glacial acetic acid, and the radioactivity was measured in a liquid scintillation counter, LSC-1000 (Aloka Co., Ltd., Tokyo). On the other hand, when [$^{125}$I]E-2078 was infused, the $^{125}$I-radioactivity in the dissected hemisphere of the perfused brain was counted in a gamma-counter, ARC-605 (Aloka Co., Ltd., Tokyo).

### Capillary Depletion Study

A capillary depletion technique (8) modified by Triguero *et al.* (9) was used. Briefly, after [$^{125}$I]E-2078 was perfused, the perfused brain was homogenized in buffer A (103 m*M* NaCl, 4.7 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.2 m*M* $KH_2PO_4$, 1.2 m*M* $MgSO_4$, 15 m*M* Hepes, 25 m*M* $NaHCO_3$, 10 m*M* D-glucose, 1 m*M* pyruvic acid, and 0.5% BSA). An equal volume of 26% dextran dissolved in buffer A without $NaHCO_3$, D-glucose, pyruvic acid, and BSA was added to the brain homogenate and centrifuged for 10 min at 6000*g* and 4°C. The radioactivities of the pellet and supernatant were measured in a gamma-counter, as the capillary and parenchyma fractions, respectively.

### Brain Microdialysis Study

The brain microdialysis was carried out with a method reported previously (12). The transcranial type of brain microdialysis cannula was constructed from a dialysis fiber (Lento H.F., Organon Technica Corp., OK) and 33-gauge stainless-steel tubing (Hypodermic Type 304, Small Parts Corp., Miami, FL) under a dissecting microscope (SZH-ILLD, Olympus Optical Co. Ltd., Tokyo). The molecular weight cutoff of the dialysis fiber is 12,500 and the inner diameter of the dialysis fiber is 200 μm. With both ends of a 22-mm dialysis fiber, 30- and 25-mm stainless-steel tubings were connected using surgical adhesive (Aron Alpha A, Sankyo Co. Ltd., Tokyo). The distance between the stainless-steel tubing (i.e., the area where the dialysis is intended to take place) was 8 mm. The 25-mm stainless-steel tubing was linked with polyethylene tubing (SP-10, Natsume Seisakusho Ltd., Tokyo) for dialysate infusion. To perfuse Ringer's–Hepes buffer (RHB; 141 m*M* NaCl, 4.0 m*M* KCl, 2.8 m*M* $CaCl_2$, and 10 m*M* Hepes, pH 7.4) containing 0.1% BSA through the microdialysis cannula at a constant flow rate, a 5.0-ml Luer-Lok tip 2141 syringe (Beckton-Dickinson, Rutherford, NJ) and a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA) were employed. The microdialysis cannula was implanted in the hippocampus of ketamine (235 mg/kg, i.m.)- and xylazine (2.3 mg/kg, i.m.)-anesthetized rats (Fig. 2). The rat with the surgical operation of brain perfusion was placed in a stereotaxic frame (KN-398, Natsume Seisakusyo Ltd.) and 1.0-mm holes were made on both sides of the skull 3.4 mm posterior

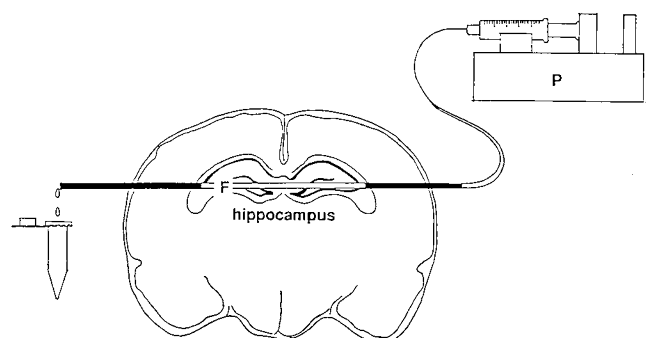


**Fig. 2.** Schematic representation of the brain microdialysis apparatus. F and P represent the microdialysis fiber and the Harvard constant infusion pump, respectively. The dialysis flow rate was 2.5 μl/min. The brain single perfusion at a rate of 1.0 ml/min via the internal carotid artery was performed with brain microdialysis.

to the bregma and 3.5 mm below the dura using a dental drill (JP-30, Takamiya Dental MFG Ltd., Tokyo). The microdialysis cannula was passed through the holes perfusing RHB into the microdialysis cannula at a constant flow rate of 2.5 μl/min. After 30 min of probe implantation, the oxygenated perfusion buffer (141 m*M* NaCl, 4.0 m*M* KCl, 2.8 m*M* $CaCl_2$, 1.0 m*M* $MgSO_4$, 10 m*M* Hepes, 10 m*M* D-glucose, and 0.1% BSA, pH 7.4) containing 50% rat blood and 5 μCi/ml [$^{125}$I]E-2078 or 2.5 μCi/ml [$^{14}$C]sucrose was perfused through the catheter at the rate of 1.0 ml/min as described above (see *In Situ* Brain Perfusion Study). Immediately, the dialysate was continuously collected for 10 min. At the end of dialysis, the cerebrospinal fluid (CSF) was obtained by the cisternal puncture technique (14). The concentration in the interstitial fluid (ISF) was determined from that in the dialysate using the relative recovery of dialysis fiber determined by the *in vitro* experiment.

### *In Vitro* Dialysis Study

The reservoir solution consisted of RHB (pH 7.4) with 0.1% BSA and 1 μCi/ml of [$^{125}$I]E-2078 or 0.25 μCi/ml of [$^{14}$C]sucrose. The dialysis fiber was put into 20 ml of reservoir solution at 37°C. The dialysis was carried out with the same solution of the reservoir at 2.5 μl/min. The dialysate was collected every 10 min and counted in a liquid scintillation counter. The *in vitro* relative recovery was estimated from the dialysate-to-reservoir concentration ratio.

### High-Performance Liquid Chromatographic (HPLC) Analysis

After brain microdialysis, the dialysate was applied to an HPLC system. The constant-flow solvent delivery system, LC-6A (Shimadzu Corp., Kyoto, Japan), was equipped with an ultraviolet detector, SPO-6A (Shimadzu Corp.), and a gradient programmer, SCL-6A (Shimadzu Corp.). A guard column, $C_{18}$ CORASIL (Waters Associations, Inc., Milford, MA), was placed between the injector and a reversed-phase analytical column, μ-Bondapak $C_{18}$ (30 cm × 3.9-mm i.d.; Waters Associations, Inc.). The mobile phase consisted of mixtures of two solvents, i.e., solvent A—10% (v/v) acetonitrile and 0.065% (v/v) trifluoroacetic acid, and solvent B—60% (v/v) acetonitrile and 0.065% (v/v) trifluoroacetic acid. The loaded samples were eluted with a linear gradient of solvent B from 20 to 50% within 15 min, followed by a linear gradient of solvent B from 50 to 100% within next 5 min. The solvent flow rate was 1.5 ml/min. The column and solvents were kept at room temperature. The $^{125}$I-radioactivity in each effluent fraction (0.75 ml) was counted in a gamma-counter.

### Data Calculation

The apparent volumes of distribution ($V_{d,app}$) in the capillary and parenchyma fractions were estimated as follows:

$$V_{d,app}(\mu l/g\ \text{brain}) = \frac{\text{dpm in capillary fraction or parenchyma fraction/g brain}}{\text{dpm}/\mu l\ \text{in perfusate}}$$

The concentration ratios of the ISF to the perfusate of [$^{125}$I]E-2078 and [$^{14}$C]sucrose were calculated as follows:

$$\text{ISF/perfusate} = \frac{\textit{in vivo}\ \text{dialysate concentration (dpm/ml)}/\textit{in vitro}\ \text{relative recovery}}{\text{perfusate concentration (dpm/ml)}}$$

## RESULTS

### Effect of Washing on the Apparent Vascular Volume

To prevent the contamination of the radioactivity remaining in the vascular space after the brain perfusion, the brain vascular space was washed out using the perfusion solution without a radiolabeled compound. The washing effect was determined using [$^3$H]inulin as a vascular space marker. In Table I, the apparent vascular volume of [$^3$H]inulin after 2 min of brain perfusion and 2 min of washing was compared to that determined without washing.

### Effect of Brain Perfusion Time and Unlabeled E-2078 on the Apparent Vascular Volume

The apparent vascular volume with washing was also compared between 2-min and 20-min brain perfusion. As listed in Table I, no significant difference in the apparent vascular volume was observed between 2-min and 20-min perfusion. Moreover, the apparent vascular volume was also compared between the presence and the absence of 2 μ*M* E-2078 in the perfusate in Table I. There was no significant difference in the apparent vascular volume between the presence and the absence of 2 μ*M* E-2078 in the perfusion solution.

### Capillary Depletion Study

Figure 3 illustrates the time courses of the apparent distribution volume of [$^{125}$I]E-2078 for the brain parenchyma and capillary determined by the capillary depletion technique combined with the brain perfusion. A remarkable time-dependent increase in the distribution volume of [$^{125}$I]E-2078 was observed for the brain parenchyma. The

**Table I.** Effect of Washing Time, Perfusion Time, and Unlabeled E-2078 on the Apparent Vascular Volume

| Perfusion time (min) | Apparent vascular volume per g brain[a] (μl/g brain) | |
|---|---|---|
| | Absence of E-2078 | Presence of E-2078[b] |
| 2[c] | 2.96 ± 0.05 | n.d.[d] |
| 2[e] | 0.797 ± 0.034* | n.d. |
| 20[e] | 0.994 ± 0.138 | 0.919 ± 0.045 |

[a] [$^3$H]Inulin was used as the vascular marker of the brain. The values are the mean ± SE of three rats.
[b] Brain perfusion of [$^3$H]inulin was performed in the presence of 2 μ*M* E-2078.
[c] After brain perfusion, the apparent vascular volume was determined without washing of the vascular space.
[d] Not determined.
[e] After brain perfusion, the vascular space was washed for 2 min by use of perfusion without radioisotope.
* Significantly different from that without washing of the vascular space at $P < 0.05$ by Student's *t* test.

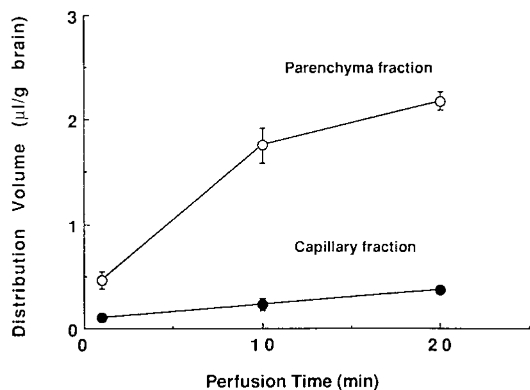


**Fig. 3.** Time courses of [$^{125}$I]E-2078 uptake into brain parenchyma and capillary by *in situ* brain perfusion technique in rats. After perfusion of [$^{125}$I]E-2078 for 20 min at the rate of 1.0 ml/min followed by perfusion of washing solution for 2 min at the same rate, the rat was decapitated and the ipsilateral hemisphere to the injected side was dissected and homogenized. An equal volume of 26% dextran was added to the brain homogenate and the mixture was centrifuged for 10 min at 6000*g*. The radioactivities of the pellet and supernatant were measured as capillary fraction and parenchyma fraction, respectively. The uptake into parenchyma (○) and capillary (●) is expressed in terms of the apparent volume of distribution. Each point represents the mean ± SE of three or four experiments.

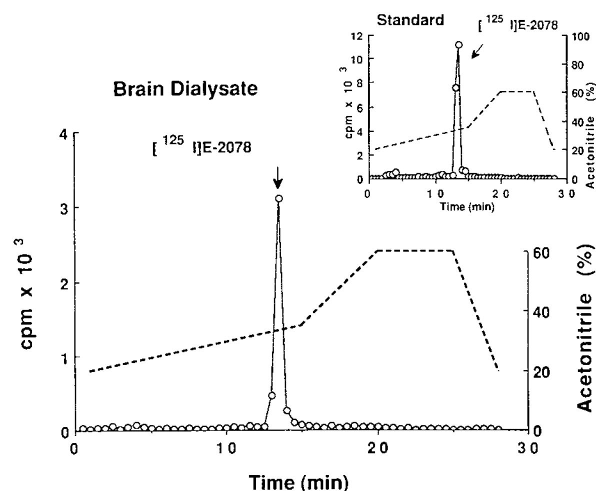


**Fig. 4.** HPLC profile of [$^{125}$I]E-2078 in the dialysate during brain perfusion of [$^{125}$I]E-2078 for 10 min at the rate of 1.0 ml/min. The dialysate was injected onto the reversed-phase HPLC column (µ-Bondapak $C_{18}$). The solvents used were mixtures of water and acetonitrile containing 0.065% trifluoroacetic acid. The loaded sample was eluted with a linear gradient of acetonitrile from 20 to 35% within 15 min, followed by a linear gradient of acetonitrile from 35 to 60% within the next 5 min (dashed line). The radioactivity in each eluent (0.75 ml) was determined. An HPLC profile of a standard sample of [$^{125}$I]E-2078 is illustrated in the inset.

distribution volume of [$^{125}$I]E-2078 in the brain parenchyma after 20-min perfusion was 2.18 ± 0.09 µl/g brain (mean ± SE; $n$ = 3) and was sixfold greater than that in the brain capillary (0.367 ± 0.030 µl/g brain). Comparing the total distribution volume of [$^{125}$I]E-2078 for the brain after 20-min perfusion, i.e., 2.54 ± 0.08 µl/g brain (Fig. 3), to that of [$^3$H]inulin (Table I), a threefold greater value was observed for [$^{125}$I]E-2078 than for [$^3$H]inulin.

### Effect of L-Tyrosine on the Distribution Volume of [$^{125}$I]E-2078 in the Brain Parenchyma

The effect of L-tyrosine on the transport of [$^{125}$I]E-2078 into the brain parenchyma after 10-min brain perfusion with 2-min washing of the vascular space was also examined. The apparent distribution volumes of the brain parenchyma fraction of [$^{125}$I]E-2078 in the absence and the presence of 5 m*M* L-tyrosine were 1.76 ± 0.17 µl/g brain (mean ± SE of four experiments) and 2.39 ± 0.07 µl/g brain (mean ± SE of three experiments), respectively.

### Brain Microdialysis Study

Figure 4 illustrates the HPLC profile of [$^{125}$I]E-2078 in the dialysate after brain microdialysis performed with brain perfusion for 10 min. The chromatogram of the dialysate was very similar to that of authentic [$^{125}$I]E-2078 (shown in the inset in Fig. 4), whereas some peaks of its metabolites were obviously observed in the chromatogram of [$^{125}$I]E-2078 incubated with brain homogenate at 37°C for 60 min (data not shown).

The *in vitro* dialysate-to-reservoir concentration ratios of [$^{125}$I]E-2078 and [$^{14}$C]sucrose were determined to be 3.35 × $10^{-2}$ ± 0.53 × $10^{-2}$ and 1.80 × $10^{-1}$ ± 0.29 × $10^{-1}$, respectively. Using these *in vitro* concentration ratios, the brain ISF were estimated for [$^{125}$I]E-2078 and [$^{14}$C]sucrose from the dialysate concentrations obtained in brain microdialysis experiments (see Materials and Methods). The concentration ratio of the ISF to the perfusate was compared between [$^{125}$I]E-2078 and [$^{14}$C]sucrose in Table II. Moreover, the concentration ratio of the CSF to the perfusate was also compared between [$^{125}$I]E-2078 and [$^{14}$C]sucrose. As listed in Table II, the concentration ratio of [$^{125}$I]E-2078 between the ISF and the perfusate was approximately 100-fold greater than that of [$^{14}$C]sucrose, whereas the concentration ratio of [$^{125}$I]E-2078 between the CSF and the perfusate was not significantly different from that of [$^{14}$C]sucrose.

## DISCUSSION

So far, the peptide delivery to the central nervous system is considered to be very difficult, because the BBB (i.e.,

**Table II.** Comparison of Concentration Ratio of Interstitial Fluid to Perfusate and Cerebrospinal Fluid to Perfusate Between [$^{125}$I]E-2078 and [$^{14}$C]Sucrose Determined by Brain Microdialysis

| | Concentration ratio[a] | |
|---|---|---|
| | [$^{125}$I]E-2078 | [$^{14}$C]Sucrose |
| ISF/perfusate | 2.92 × $10^{-1}$ ± 0.50 × $10^{-1}$ | 2.71 × $10^{-3}$ ± 1.43 × $10^{-3}$ |
| CSF/perfusate | 2.96 × $10^{-3}$ ± 0.26 × $10^{-3}$ | 1.32 × $10^{-3}$ ± 0.43 × $10^{-3}$ |

[a] Brain microdialysis was performed with brain perfusion for 10 min. The concentrations of [$^{125}$I]E-2078 and [$^{14}$C]sucrose in the ISF were determined using the respective *in vitro* recoveries by the analysis of the brain dialysate. The values are the mean ± SE of three rats.

brain capillary endothelial cells) strictly selects the penetration of macromolecules from the circulating blood to the ISF of the brain parenchyma (15). Moreover, enzymatic instability of native peptides in the systemic circulation would also make it difficult to deliver a neuropeptide as a central acting pharmaceutical (16). E-2078, a synthetic dynorphin analogue peptide, is a promising candidate which has an analgesic effect after the systemic administration (10,11). In a previous study using *in vitro* isolated bovine brain capillaries (3), we have clarified that E-2078 is taken up by the brain capillaries via absorptive-mediated endocytosis. Since the surface area of the BBB is 5000-fold greater than that of the blood–CSF barrier (BCSF barrier) (17), transcytosis through the BBB could be postulated to play a dominant role for the penetration of E-2078 into the brain. To prove this hypothesis, a comparative study for the penetration of E-2078 would be necessary between the brain ISF and the CSF. Hence, the capillary depletion and brain microdialysis techniques were employed in the present study.

As shown in Fig. 3, a significant time dependence was observed for the distribution volume of [$^{125}$I]E-2078 in the brain parenchyma, while no time dependence was observed for the apparent vascular volume, suggesting penetration of [$^{125}$I]E-2078 into the brain parenchyma under *in vivo* conditions. During separation of the parenchyma and capillary, 2-min washing after the brain perfusion was found to be effective to decrease the contamination of the residual perfusate in the vascular space to the parenchyma. Additionally, the contamination of the capillary to the brain parenchyma fraction is reported to be less than 1% during the capillary precipitation experiment (8). Generally, exocytosis of the peptide from the intracellular fluid is energy and temperature dependent. Since the isolation procedure was carried out at 4°C, the release of [$^{125}$I]E-2078 from the capillary endothelial cells would be negligible during the isolation procedure. Therefore, the data shown in Fig. 2 should not represent contamination of the capillaries or release of the peptide from the endothelial cells, but the penetration of [$^{125}$I]E-2078 across the BBB.

The distribution volume of [$^{125}$I]E-2078 in parenchyma fraction at 10 min (Fig. 2) is severalfold smaller than the value reported for cationized albumin (9), whereas both the basic peptides E-2078 and cationized albumin were reported to be transported via absorptive-mediated endocytosis at the BBB (1,3). The present perfusion studies were performed without cauterization of the principal tributaries of the internal carotid artery, while they were cauterized in a previous study (9). Therefore, the lower distribution volume of [$^{125}$I]E-2078 could be ascribed to the lower availability of the perfusate to the cerebral blood flow.

As E-2078 is a basic peptide of isoelectric point 10.0 (unpublished data), the restrictive nature of the capillary might be changed by the interaction of the basic peptide to the surface of the plasma membrane. After intramuscular administration of E-2078 to rats at a dose of 0.5 mg/kg, enough for its analgesic effect, the peak plasma concentration of E-2078 was approximately 0.3 $\mu M$ (unpublished data), which is less than 2 $\mu M$. The absence of a significant effect of 2 $\mu M$ E-2078 on the vascular volume of the brain capillaries (Table I) supports that E-2078 would not cause enhancement of the nonspecific permeability of the macromolecule through the brain capillary during systemic circulation. Accordingly, the time-dependent increase in the distribution volume of [$^{125}$I]E-2078 for the brain parenchyma would be attributed to absorptive-mediated endocytosis in the capillary endothelial cells as suggested previously using *in vitro* isolated bovine brain capillary (3).

Since L-tyrosine can be transported via a carrier-mediated system at the BBB (17), the transport of degradation products such as [$^{125}$I]tyrosine would cause an overestimation of the transport of [$^{125}$I]E-2078 to the brain. In contrast to [tyrosyl-$^{3}$H]leucine enkephalin (16), [$^{125}$I]E-2078 is suggested to be transported as the intact form through the BBB, because L-tyrosine largely did not affect the apparent distribution volume of [$^{125}$I]E-2078 (see Results) and no metabolites of [$^{125}$I]E-2078 were eluted on HPLC from the brain dialysate (Fig. 4). These results agree well with the previous study (3) using the *in vivo* brain uptake method and the *in vitro* isolated bovine brain capillary uptake method, which suggested no significant metabolism of [$^{125}$I]E-2078 in the brain capillary.

In order to prove our hypothesis regarding the pathway for the penetration of E-2078 into the brain, measurement of the peptide in the brain ISF would provide direct *in vivo* evidence. In the previous study (12), we demonstrated the brain microdialysis technique as a novel experimental procedure for determination of the endogenous choline level in the ISF of the brain parenchyma. Hence, the brain microdialysis technique was applied to determine the concentration of [$^{125}$I]E-2078 in the brain ISF under physiological conditions. As the hypocampus is near the cortex, which is a capillary-rich region in the brain, the transcranial microdialysis fiber was implanted in the hypocampus to determine the concentration in the brain ISF (Fig. 2).

[$^{14}$C]Sucrose, a capillary nonpermeable marker, has been used to evaluate damage effects of the fiber implantation on the BBB. The low ISF-to-perfusate concentration ratio of [$^{14}$C]sucrose (Table II) suggests that nonspecific permeation of the perfusate through the brain capillary is very minor after implantation of the microdialysis fiber. Additionally, it has been reported that the implantation of a horizontal fiber would not cause a significant effect on the transport of $\alpha$-aminoisobutyrate (7), which is taken up relatively faster than [$^{125}$I]E-2078 at the BBB. Accordingly, the brain microdialysis technique has been demonstrated to be a useful method to determine the brain interstitial level for a substrate which would be slowly transported through the BBB and/or rapidly metabolized in the brain parenchyma.

As clearly shown in Table II, a 100-fold greater concentration ratio of [$^{125}$I]E-2078 between the ISF and the perfusate in comparison with that of [$^{14}$C]sucrose has provided direct evidence for significant *in vivo* transport of [$^{125}$I]E-2078 from the vascular lumen to the brain ISF. Moreover, the concentration ratio of [$^{125}$I]E-2078 between the CSF and the perfusate was shown to be approximately 100-fold smaller than that between the ISF and the perfusate and also shown to be not significantly different from that of [$^{14}$C]sucrose, demonstrating that the transport of [$^{125}$I]E-2078 into the brain is dominantly ascribed to the BBB transport but not to the BCSF barrier. The very low concentration of [$^{125}$I]E-2078 in the CSF (Table II) suggests the lack of a transport system for E-2078 at the BCSF barrier. How-

ever, as [$^{125}$I]E-2078 might diffuse from the brain interstitial fluid to the CSF to some extent, the concentration of [$^{125}$I]E-2078 in the CSF might gradually increase at later times.

In order to determine the concentration of [$^{125}$I]E-2078 in the ISF, we have used the concentration in the dialysate and the *in vitro* concentration ratio between dialysate and reservoir assuming the same permeability between *in vivo* and *in vitro* conditions. Although anatomical factors such as tortuosity of the brain cells and the extracellular fluid volume of the brain have been suggested to influence *in vivo* diffusion in the ISF (18), there seems to be no practical method at the present time to correct the difference between diffusion *in vivo* and diffusion *in vitro*. Further elaborate study would be necessary to clarify this issue.

In conclusion, using capillary precipitation and brain microdialysis techniques, we have successfully confirmed our hypothesis postulated in the previous study, that E-2078 can cross the BBB *in vivo* as the intact form and that the transport through the BBB is a major pathway to the delivery of E-2078 into the brain. The brain microdialysis technique was demonstrated to be a useful strategy to prove *in vivo* transport of relatively low-permeable substrates such as neuropeptides through the BBB.

## ACKNOWLEDGMENTS

The authors greatly appreciate Professor William M. Pardridge for his valuable discussions. The authors thank Miss Sachiko Kawasaki and Yuko Kasama for their excellent technical assistance of brain microdialysis study, Drs. Yasuo Miyake and Teruaki Yuzuriha, Research & Development Division, Eisai Co., Ltd., for kindly supplying us with unlabeled and [$^{125}$I]E-2078. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, the Project Research Fund from the Graduate School of Natural Science and Technology, Kanazawa University, the Naito Foundation, the Nakatomi Foundation, and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

## REFERENCES

1. A. K. Kumagai, J. B. Eisenberg, and W. M. Pardridge. Absorptive-mediated endocytosis of cationized albumin and a β-endorphin-cationized albumin chimeric peptide by isolated brain capillaries: Model system of blood-brain barrier transport. *J. Biol. Chem.* 262:15214–15219 (1987).
2. K. R. Duffy, W. M. Pardridge, and R. G. Rosenfeld. Human blood-brain barrier insulin-like growth factor receptor. *Metabolism* 37:136–140 (1988).
3. T. Terasaki, K. Hirai, H. Sato, Y. S. Kang, and A. Tsuji. Absorptive-mediated endocytosis of a dynorphin-like analgesic peptide, E-2078, into the blood-brain barrier. *J. Pharmacol. Exp. Ther.* 251:351–357 (1989).
4. W. H. Oldendorf. Measurement of brain uptake of radiolabeled substances using a tritiated water internal standard. *Brain Res.* 24:372–376 (1970).
5. U. Ungerstedt, M. Herrera-Marschitz, U. Jungnelius, L. Stahle, U. Tossman, and T. Zetterström. Dopamine synaptic mechanisms reflected in studies combining behavioral recordings and brain dialysis. *Adv. Biosci.* 37:219–231 (1982).
6. Y. Takasato, S. I. Rapoport, and Q. R. Smith. An *in situ* brain perfusion technique to study cerebrovascular transport in the rat. *Am. J. Physiol.* 247:H484–H493 (1984).
7. H. Benveniste, J. Drejer, A. Schousboe, and N. H. Diemer. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* 43:1369–1374 (1984).
8. J. B. Fishman, J. B. Rubin, J. V. Handrahan, J. R. Conner, and R. E. Fine. Receptor-mediated transcytosis of transferrin across the blood-brain barrier. *J. Neurosci. Res.* 18:299–304 (1987).
9. D. Triguero, J. Buciak, and W. M. Pardridge. Capillary depletion method for quantification of blood-brain barrier transport of circulating peptides and plasma proteins. *J. Neurochem.* 54:1882–1888 (1990).
10. S. Tachibana, H. Yoshino, Y. Arakawa, T. Nakazawa, S. Araki, T. Kaneko, and K. Yamatsu. A novel analogue (E-2078) of dynorphin (1): Design and structure-activity relationship. *Int. Narcotics Res. Conf.*, 1987, p. 60.
11. T. Nakazawa, Y. Furuya, T. Kaneko, K. Yamatsu, H. Yoshino, and S. Tachibana. Analgesia produced by E-2078, a systemically active dynorphin analog, in mice. *J. Pharmacol. Exp. Ther.* 252:1247–1254 (1990).
12. Y. S. Kang, T. Terasaki, and A. Tsuji. Dysfunction of choline transport system through blood-brain barrier in stroke-prone spontaneously hypertensive rats. *J. Pharmacobio-Dyn.* 13:10–19 (1990).
13. W. M. Pardridge and G. Fierer. Blood-brain barrier transport of butanol and water relative to *N*-isopropyl-*p*-iodoamphetamine as the internal reference. *J. Cereb. Blood Flow Metab.* 5:275–281 (1985).
14. R. C. Chou and G. Levy. Effect of heparin or salicylate infusion on serum protein binding and on concentrations of phenytoin in serum, brain and cerebrospinal fluid of rats. *J. Pharmacol. Exp. Ther.* 219:42–48 (1981).
15. W. M. Pardridge. Receptor-mediated peptide transport through the blood-brain barrier. *Endocrine Rev.* 7:314–330 (1986).
16. W. M. Pardridge and L. J. Mietus. Enkephalin and blood-brain barrier: Studies of binding and degradation in isolated brain microvessels. *Endocrinology* 109:1138–1143 (1981).
17. W. M. Pardridge. Brain metabolism: A perspective from the blood-brain barrier. *Physiol. Rev.* 63:1481–1535 (1983).
18. H. Benveniste, A. J. Hansen, and N. S. Ottosen. Determination of brain interstitial concentrations by microdialysis. *J. Neurochem.* 52:1741–1750 (1989).