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Dynorphin A (1–8) Analog, E-2078, Crosses the Blood-Brain Barrier in Rhesus Monkeys[1]

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ABSTRACT

E-2078 is a dynorphin A (1–8) analog, [N-methyl-Tyr[1], N-methyl-Arg[7]-D-Leu[8]] dynorphin A (1–8) ethylamide. Its ability to cross the blood-brain barrier was examined in rhesus monkeys using matrix-assisted laser desorption/ionization mass spectrometry. *In vivo* studies were carried out by i.v. injecting E-2078, 10 mg/kg, a dose that had been found to be antinociceptive, to rhesus monkeys. Blood and cerebrospinal fluid samples were collected at various time points after the injection. It was found that E-2078 was stable *in vivo* in rhesus monkey blood. No biotransformation products were detected in the blood. Mass spectrometric analysis of the cerebrospinal fluid samples collected after E-2078 injection detected the presence of E-2078, indicating that E-2078 had crossed the blood-brain barrier. These findings are consistent with the possibility that systemically administered E-2078 could produce centrally mediated behavioral and physiological effects.

Studies of endogenous neuropeptide dynorphin A (1–17) have indicated that this peptide and its congeners may attenuate the behavioral symptoms of the opioid withdrawal syndrome in morphine-dependent rodents and may be useful as a therapeutic agent for the treatment of opioid dependency in man (Takemori *et al.*, 1992, 1993). It has also been shown that dynorphin A peptides have analgesic properties and may have potential application in the management of pain (Hooke and Lee, 1995; Smith and Lee, 1988). However, natural dynorphin A peptides are susceptible to enzymatic degradation *in vitro* and *in vivo* in a biological system (Goldstein *et al.*, 1979; Young *et al.*, 1987). Dynorphin A peptides undergo proteolytic cleavages in a biological matrix to form a variety of biotransformation products (Butelman *et al.*, 1996; Chou *et al.*, 1994a, 1994b; Yu *et al.*, 1996a).

E-2078, code-named for [N-methyl-Tyr[1], N-methyl-Arg[7]-D-Leu[8]] dynorphin A (1–8) ethylamide, is a dynorphin A (1–8) analog (Tachibana *et al.*, 1988; Yoshino *et al.*, 1990). Studies in our laboratory and by others have indicated that this synthetic peptide is stable against enzymatic cleavages in biological matrices (Nakazawa *et al.*, 1990; Yu *et al.*, 1996b). Modification with a methyl group at Tyr[1], N-methyl-Tyr[1], effectively protected the cleavage at the N-terminal Tyr[1] position. Peptide linkage of Arg(6)-Arg(7) is usually the other site of cleavage of dynorphin. A peptides in a biological matrix (Silberring *et al.*, 1992; Yu *et al.*, 1996a). Modification with a methyl group at the N-Arg[7] position effectively blocked this biotransformation. E-2078 binds to κ-opioid receptors just like dynorphin A (1–17) as studied *in vitro* with isolated organ preparations: guinea pig ileum, mouse vas deferens and rabbit vas deferens (Yoshino *et al.*, 1990). Intravenously administered E-2078 was approximately equipotent to morphine in the tail-pinch assay in mice (Yoshino *et al.*, 1990). This systemic effectiveness of E-2078 was attributed to its stability against enzymatic degradation (Nakazawa *et al.*, 1990; Yoshino *et al.*, 1990). In preliminary studies in humans, E-2078 exhibited analgesic properties when used in patients with severe pain after lower abdominal surgery in clinical studies (Fujimoto and Momose, 1995; Tachibana, 1996). Our previous studies of the biotransformation of this Dyn A (1–8) analog indicated that E-2078 was stable *in vitro* both in human and rhesus monkey blood, and *in vivo* in rhesus monkey blood. No major biotransformation products were detected (Yu *et al.*, 1997). Studies reported by others have shown that E-2078 had effects that might be through action on the central nervous systems after systemic administration, including thermal antinociception in rhesus monkeys (E. R. Butelman, J. A. Vivian, J. H. Woods, unpublished observations) and water diuresis in humans (Ohnishi *et al.*, 1994). We, therefore, hypothesized that E-2078 might be able

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ABBREVIATIONS: 4HCCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; Arg, arginine; Tyr, tyrosine; Leu, leucine; CSF, cerebrospinal fluid; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; RBC, red blood cell count.

to cross the blood-brain barrier after systemic administration and thus be available for central as well as peripheral sites of action. *In vitro* studies using isolated bovine brain capillaries (Terasaki *et al.*, 1989) and *in vivo* microdialysis studies in Wistar rats (Terasaki *et al.*, 1991), in which measuring the radioactivity from the radioisotope labeled E-2078 was measured, have indicated that [^{125}I]E-2078 was absorbed into and crossed the blood-brain barrier.

E-2078 (0.18–0.56 mg/kg, s.c.), similar to nonpeptidic *kappa* agonists, is also active as a diuretic in rhesus monkeys (Dykstra *et al.*, 1987; Vivian *et al.*, 1995). Furthermore, the presently studied E-2078 does, 10 mg/kg, also caused thermal antinociception in rhesus monkeys (E. R. Butelman, J. A. Vivian, J. H. Woods, unpublished observations). The aim of our studies was to determine whether i.v. administered E-2078 at a behaviorally active dose could be detected in CSF in rhesus monkeys. Such a finding would be consistent with the notion that this peripherally administered peptide could exert behavioral effects by acting on the central nervous system. This could make E-2078 an especially attractive pharmacological tool for the study of recently reported effects of *kappa* agonists and dynorphins, namely in the fields of analgesia (Hooke and Lee, 1995), opioid dependence and withdrawal (Takemori *et al.*, 1992, 1993) and cocaine abuse (Claye *et al.*, 1997; Shippenberg *et al.*, 1996; Spangler *et al.*, 1996; Unterwald *et al.*, 1994). Many signs and symptoms of opioid withdrawal are thought to be mediated primarily by central mechanisms (Maldonado *et al.*, 1992), whereas analgesic or antinociceptive effects of opioids can be either centrally or peripherally mediated, depending on the experimental situation (Gmerek *et al.*, 1986; Stein *et al.*, 1989). It is thought that the primary action of cocaine in drug abuse, as well as the molecular abnormalities caused by cocaine in experimental animals, are mediated in specific brain regions, thus probably requiring a therapeutic intervention that is centrally targeted (Di Chiara and Imperato, 1988, Spangler *et al.*, 1996; Unterwald *et al.*, 1994).

The general concept that some peptides can cross the blood-brain barrier as intact molecules has gained more acceptance in recent years (Banks *et al.*, 1992). Studies have shown in both *in vitro* and *in vivo* systems that peptides can cross the blood-brain barrier, using a variety of direct techniques such as brain perfusion methods, brain microdialysis, specific radioimmunoassay, high-performance liquid chromatography, as well as indirect mathematical procedures (Banks and Kastin, 1990). The recent development of MALDI MS provides with sensitive and specific analytical technique for peptide detection and identification. Its tolerance to biological impurities in the samples allows minimal sample pretreatment, which makes MALDI MS an ideal technique for detection of peptide passage of the blood-brain barrier. We provide direct and specific evidence to demonstrate that intact E-2078 crosses the blood-brain barrier during *in vivo* studies on rhesus monkeys.

Materials and Methods

Subjects. Five adult rhesus monkeys, *Macaca mulatta*, were used in the *in vivo* studies. Two monkeys, one male, 12 kg, 16 yr; and one female, 7.5 kg, 18 yr were used for the *in vivo* qualitative studies. Three monkeys, one male, 9.4 kg, 8 yr; two female, 6 kg and 6.5 kg, 18 and 9 yr, respectively, were used for the *in vivo* quantitative studies. These monkeys were housed singly with free access to water, and were fed approximately 30 Purina monkey chow biscuits (St. Louis, MO) daily and fresh fruit twice per week. The monkeys were housed on a 12:12 light:dark cycle (light on at 7:00). Experiments were carried out between 10:00 and 15:00.

Chemicals. [N-methyl-Tyr^1, N-methyl-Arg^7-D-Leu^8] Dyn A (1–8) ethylamide (E-2078) was synthesized and kindly supplied by Eisai Co. Ltd. (Ibaraki, Japan). Dyn A (1–8) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Diphenhydramine HCl was purchased from Sigma Chemical Company (St. Louis, MO). Ketamine HCl was obtained from Fort Dodge (Fort Dodge, IA). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ). Saline (0.9% NaCl) and dextrose were from Abbott Laboratories (North Chicago, IL). 4HCCA was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). High performance liquid chromatography grade acetonitrile was purchased from Burdick & Jackson (Muskegon, MI) and TFA from Fisher Scientific (Fair Lawn, NJ).

E-2078 *in vivo* studies in rhesus monkeys. Monkeys received injections with diphenhydramine HCl (1.2 mg/kg, i.m.) as a pretreatment to limit the possible consequences of histamine release following administration of relatively large amounts of E-2078. Thirty min later, they were anesthetized with ketamine HCl (10 mg/kg, i.m.). The back of the lower leg area and the dorsal upper neck/lower skull area were carefully shaved to avoid any adventitious bleeding. An indwelling catheter (Angiocath, 22 gauge, 1 inch long, Becton Dickinson, Sandy, UT) was acutely placed in a superficial vein of each leg, secured and flushed with heparinized saline (20 U/ml), and connected to a multisample injection port. One milliter of blood sample was obtained as control and placed in a 2-ml vacutainer preconditioned with EDTA on ice. All blood sampling was followed by port and catheter flushing with heparinized saline. The animal was then placed on a heating pad (37°C) on a surgery table. At the beginning of the studies, a spinal needle (22 gauge, 1.5 inch long, Becton Dickinson, Franklin Lakes, NJ) was carefully inserted in the cisterna magna, by puncturing the skin and atlanto-occipital membrane. Before E-2078 injection, the red blood cell count of the CSF from three rhesus monkey subjects participated in the quantitative studies was examined using a hemocytometer (model 1490, Hausser Inc., Horsham, PA) under a light microscope with 400× magnification. Approximately 15 μl of CSF were collected from each monkey subject. As a comparison, the RBC of the blood from the rhesus monkeys was also examined.

During the sampling period, the animal received a dextrose/saline i.v. infusion (approximately 30 ml/kg/hr) from the catheter that had previously been used for the E-2078 injection. Supplemental ketamine HCl (approximately 5 mg/kg) was administered at hourly intervals to maintain the animal in an anesthetized state throughout the sampling period.

After obtaining a clean CSF sample as control, the required amount of E-2078 was dissolved in saline (10 mg/kg in 20 mg/ml solution). The E-2078 was dissolved in saline (10 mg/kg in 20 mg/ml solution). The E-2078 was injected in one of the leg catheters (injection time was approximately 15 sec). At the end of injection, a timer was started, and the injection catheter was flushed with heparinized saline. Sampling was performed at the following time points: CSF sampling, 3, 8, 15, 30, 60, 90, 120 and 180 min; blood sampling from the contralateral catheter only at 0, 5, 15, 30, 60, 90, 120 and 180 min. When CSF samples were to be taken, the stylet of the spinal needle was removed, and the first CSF drop exiting the spinal needle (approximately 50 μl) was discarded from each sample. A sample of CSF of 0.1 ml was removed and placed in a 2-ml cryovial containing 0.3 ml of 1% TFA. The content of the vial was gently mixed and the vial rapidly placed on ice. At all times in the experiments, only CSF samples that did not show signs of blood contamination were used. If blood contamination of the CSF samples was suspected (*i.e.*, by a slight red coloring in the sample), the spinal needle would be gently moved or removed and re-inserted until a clean sample could be obtained. Individual CSF collection time points were deleted if clear

CSF could not be obtained within a limited time interval (around 5 min). CSF samples were stored in a −40°C freezer until the time of analysis. Blood samples were centrifuged (3400 × g at 0–5°C for 5 min). A 0.2-ml aliquot of plasma was placed in a cryovial containing 1.8 ml of 1% TFA and kept at −40°C until the time of analysis.

Before analysis, the plasma samples were thawed at room temperature. To 2.0 ml of plasma sample solution was added 40 μl of 80 μM (1.54 μg/ml) Dyn A (1–8) in 0.05% aqueous TFA as the internal standard. The solution containing plasma, the peptide, the internal standard and TFA, was centrifuge-filtered with Centricon-SR3 concentrators (Amicon Inc., Beverly, MA), with molecular weight cut-off of 3000 Da. Twenty microliters of the filtrate were mixed with 10 μl of acetonitrile for analysis by mass spectrometry.

Before analysis, the CSF samples were thawed at room temperature. In the first set of studies, qualitative analysis of the CSF from two rhesus monkey subjects was performed. In the second set of studies, quantitative analysis of the CSF from the other three rhesus monkey subjects was conducted using Dyn A (1–8) as the internal standard. To 100 μl of CSF sample was added 2 μl of 8 μM (0.154 μg/ml) Dyn A (1–8) in 0.05% aqueous TFA as the internal standard. One hundred microliters of the CSF sample solution was evaporated in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY) for 35 min to reduce the total volume to approximately 40 μl. Twenty microliters of the resulting CSF sample were mixed with 10 μl of acetonitrile for analysis by mass spectrometry.

Mass spectrometry. The samples were analyzed by a mass spectrometer consisting of a matrix-assisted laser desorption ion source, coupled with a linear time-of-flight mass analyzer (MALDI MS). This instrument was constructed at the Rockefeller University (Beavis and Chait, 1989, 1990). A saturated matrix solution was prepared by dissolving excess amount (5 mg/ml) of 4HCCA in acetonitrile and 0.1% aqueous TFA [1:2 (v/v)]. For mass spectrometric measurement, the samples with acetonitrile [2:1 (v/v)] were mixed with the matrix solution. CSF or plasma samples were mixed with matrix solution in a ratio of 1:2 (v/v). A 0.5-μl aliquot of the resulting solution was applied to the mass spectrometer sample probe tip, and allowed to evaporate to dryness in the air. The sample probe was inserted into the mass spectrometer vacuum system where solvents in the sample were completely removed. After 5 min, a working pressure of approximately 10^{-7} torr was achieved. To obtain adequate statistics, *i.e.*, to achieve a signal-to-noise ratio of the peak of interests of more than 5:1, the results from 200 laser shots were added to produce each mass spectrum. In this study, one spectrum was obtained for each sample from each subject.

A standard curve was constructed using E-2078 standard solutions. The standard solutions were made by serial dilution of E-2078 in 0.05% TFA aqueous solution. A constant amount of internal standard, Dyn A (1–8) (1 μl of 136 μg/ml, or 0.136 μg), was added to 100 μl of standard solutions. The E-2078 standard solutions containing Dyn A (1–8) were each mixed with acetonitrile (2:1, v/v), and the resulting solutions were separately mixed with 4HCCA matrix solution (1:2, v/v). Half microliter aliquots of the resulting solutions were applied to the mass spectrometer sample probe tip for MALDI MS analysis. Mass spectrometric measurements were carried out in triplicate for each aliquot of the standard solutions. The ratio of the signal intensity from E-2078 to that from the internal standard, and the standard error of the triplicate measurements were calculated.

Results

Mass spectrometric analysis of the plasma samples confirmed that E-2078 was stable against enzymatic degradation in rhesus monkey blood *in vivo* just as we have previously found in *in vitro* studies (Yu *et al.*, 1996b; Yu, J.; Butelman, E. R.; Woods, J. H.; Chait, B. T.; Kreek, M. J., unpublished observations), *i.e.*, no biotransformation products from E-2078 were detected in any of the blood samples obtained in the five monkey subjects studied. Blood samples were collected at the time points, 0, 5, 15, 30, 60, 90, 120 and 180 min after E-2078 injection. Intact E-2078 was detectable in all the blood samples. No E-2078 was detected in blood 24 hr after injection. Figure 1 is a spectrum of E-2078 in a blood sample collected from a monkey 3 (TO) at 180 min time point, showing no detectable biotransformation product.

Mass spectrometric analysis of CSF from the rhesus monkeys after i.v. injection of E-2078 detected the presence of E-2078 in CSF (fig. 2) indicating that E-2078 had crossed the blood-brain barrier. CSF was sampled at the time points, 3, 8, 15, 30, 60, 90, 120 and 180 min after E-2078 injection. E-2078 was detected in CSF collected at all the time points from the five rhesus monkey subjects used in our studies. In the quantitative studies conducted in three monkey subjects, it was found that the signal intensity from E-2078 varied more in CSF than in plasma among the subjects, table 1.

Because of the nature of the matrix-assisted laser desorption/ionization processes, the use of an internal standard [Dyn A (1–8)] is essential for quantitative analysis of E-2078 (fig. 2). As shown in the calibration curve (fig. 3), the relative signal intensity is linearly correlated to the amount of peptide applied over the concentration range between 22.22 and 555.55 pg of E-2078 applied to the sample probe tip. The linear correlation coefficient of the curve fit was determined to be 0.995. The concentration of E-2078 in CSF and plasma at various time points was listed in table 1. The maximum percentage of E-2078 detected in blood relative to the total amount of the peptide injected is about 48%, calculated based on the reported value of 54 ml/kg blood volume per body weight found in rhesus monkeys (Gregersen *et al.*, 1959).

The RBC measured in the CSF from three rhesus monkeys were 0, 0.6 and 1.0 RBC/μl, whereas the RBCs of the blood from the same rhesus monkeys were 6.6, 4.7 and 4.0 million RBC/μl, respectively. These results indicated that there was a negligible amount of blood contamination in the CSF samples. Interestingly, in one monkey subject, subject 5 (J.U.), an older female adult rhesus monkey, a high white blood cell count (52 white blood cell count/μl) was observed in the CSF, in the absence of other overt signs of disease.

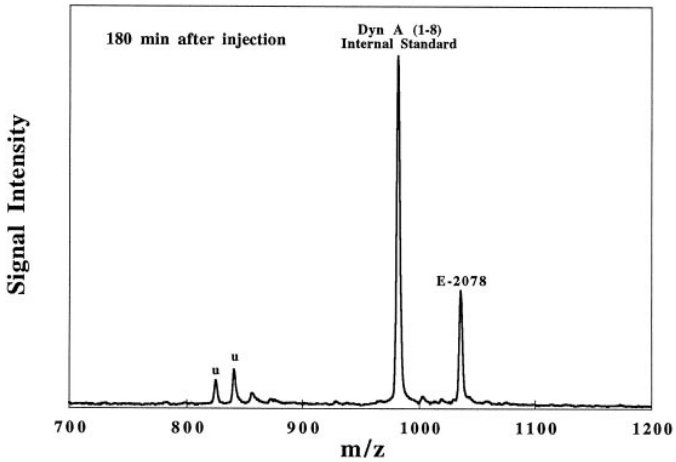

Fig. 1. Matrix-assisted laser desorption/ionization mass spectrum of blood sample collected at 180 min time point after E-2078 (10 mg/kg) i.v. injection to rhesus monkey subject 3. u, Unidentified peaks.

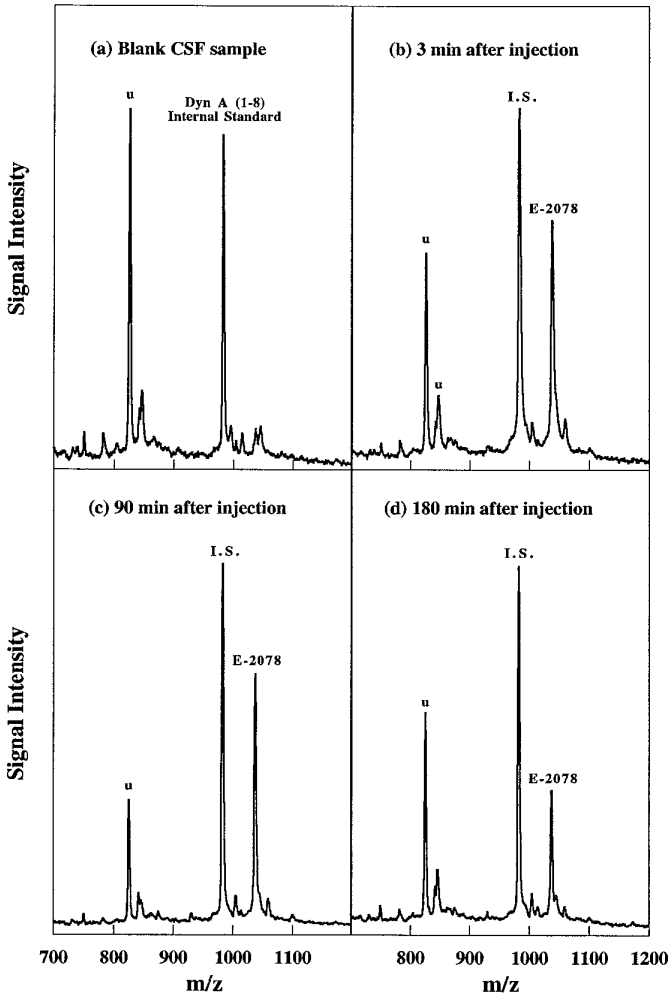

Fig. 2. Matrix-assisted laser desorption/ionization mass spectra of cerebrospinal fluid from rhesus monkey subject 5 (a) before E-2078 intravenous injection; (b) 3 min after injection; (c) 90 min after injection and (d) 180 min after injection. I.S., Internal standard, Dyn A (1–8); u, unidentified peaks.

Discussion

MALDI MS is an analytical technique developed in recent years (Hillenkamp *et al.*, 1991). It permits sensitive, simultaneous as well as specific detection of the presence of multiple peptide components in a sample. This technique tolerates biological contaminants, and requires minimal sample pretreatment. In our laboratories, this technique has been successfully applied to several studies of biotransformation of neuropeptides including Dyn A peptides in a variety of biological matrices, such as blood, brain tissues and CSF (Butelman *et al.*, 1996; Chou *et al.*, 1994a, 1994b, 1996; Yu *et al.*, 1996a, 1996b, 1996c).

The Dyn A (1–8) analog, E-2078, was stable *in vivo* in rhesus monkey blood, as was found in our previous *in vitro* and *in vivo* studies (Yu *et al.*, 1996b; Yu, J.; Butelman, E. R.; Woods, J. H.; Chait, B. T.; Kreek, M. J.; unpublished observations). No biotransformation products were detected in the blood samples. Intact E-2078 was detected in rhesus monkey CSF after E-2078 intravenous injection. E-2078 might enter the CSF by crossing the blood-CSF barrier. However, because the surface area of the blood-brain barrier (*i.e.*, cerebral capillaries) is 5000-fold greater than that of the blood-CSF barrier (mainly in circumventricular organs, such as choroid plexus) (Pardridge, 1983), transport of E-2078 through the blood-brain barrier is likely the dominant path into the CSF. The enhanced stability of E-2078 makes it more resistant to enzymatic degradation *in vivo* in monkeys, both by the enzymes present in the blood and membrane-bound enzymes. This resistance against enzymatic degradation can significantly increase the possibility for the peptide to cross the blood-brain barrier. *In vivo* studies of E-2078 in rhesus monkey did not show major biotransformation in the blood. Our recent studies (Yu *et al.*, 1997) have indicated that E-2078 had slow elimination from blood. Further studies will be needed to determine both time course and dose responses when more peptide becomes available.

The dose (10 mg/kg) of E-2078 used in the studies also produced thermal antinociception in unanesthetized rhesus monkeys (E. R. Butelman and J. H. Woods, unpublished observation). Therefore, our findings are consistent with the possibility that this peptide exerted its antinociceptive effects by acting on the receptors located in the central nervous system. These studies show that E-2078 is able to enter CSF after i.v. administration in primates, and is therefore a suitable candidate for the investigation of possible centrally mediated effects of systemically administered dynorphins.

Studies reported by other researchers have shown that [^{125}I] E-2078 crosses the blood-brain barrier, possibly through absorption-mediated endocytosis, as measured by the radioactivity from the radioisotope labeled E-2078 (Terasaki *et al.*, 1989, 1991). The authors in these studies suggested that the crossing of the blood-brain barrier was mediated by absorption. *In vitro* studies using [^{125}I] E-2078 and isolated bovine brain capillaries have shown that no significant metabolism of the labeled peptide occurred. [^{125}I] E-2078 was bound and internalized in the intact form into an osmotically reactive intracellular space (Terasaki *et al.*, 1989). The use of the opioid antagonist, naloxone, indicated that opioid receptors did not mediate the hypothesized endocytosis of [^{125}I] E-2078 through the blood-brain barrier (Terasaki *et al.*, 1989). *In vivo* studies using [^{125}I] E-2078 in male Wistar rats with microdialysis technique demonstrated the penetration of the peptide through the blood-brain barrier into the brain parenchyma. Brain interstitial fluid collected by microdialysis showed no metabolite of [^{125}I] E-2078 as measured by high performance liquid chromatographic analysis of the brain dialysate (Terasaki *et al.*, 1991).

In our studies, intact E-2078 was detected in the CSF collected after i.v. injection of E-2078 in rhesus monkey subjects. The red blood cell count measurements indicated that contamination of the CSF from blood in the sampling process is insignificant. In addition, quantitative results show that the ratio of the concentrations of E-2078 in the plasma was between 9 to 58 times of those in CSF (table 1).

In one monkey subject, subject 5 (J.U.), the CSF concentration of E-2078 appeared to be higher (approximately doubled) than in the other two subjects, Subject 3 (T.O.) and subject 4 (S.H.). Variables that could potentially affect blood-brain barrier status include viral infections (*e.g.*, simian immunodeficiency virus) (Smith *et al.*, 1994), and age (Mooradian, 1988). These variables could be considered in future studies.

In summary, Dyn A (1–8) analog, E-2078, was very stable

TABLE 1
Concentration (ng/μl) of E-2078 in CSF and plasma

Time (min)	Subject 3(TO)			Subject 4(SH)			Subject 5(JU)[a]		
	CSF	Plasma	Ratio	CSF	Plasma	Ratio	CSF	Plasma	Ratio
0[b]	N/M[c]	68	N/M	N/M	88	N/M	N/M	82	N/M
3	0.65	N/M	N/M	N/M	N/M	N/M	1.1	N/M	N/M
5	N/M	30	N/M	N/M	35	N/M	N/M	32	N/M
15	0.48	N/M	N/M	0.44	26	1:58	1.8	19	1:11
30	0.49	N/M	N/M	0.56	21	1:37	1.7	21	1:12
60	1.0	17	1:17	0.45	9.9	1:22	1.2	15	1:12
90	0.46	N/M	N/M	0.43	N/M	N/M	1.1	11	1:10
120	0.40	10	1:26	0.41	11	1:27	0.81	9.3	1:11
180	0.4	9.4	1:21	N/M	8.4	N/M	0.75	6.6	1:9

[a] See "Discussion."
[b] Speciment obtained immediately after injection.
[c] N/M, Not measured in the study.

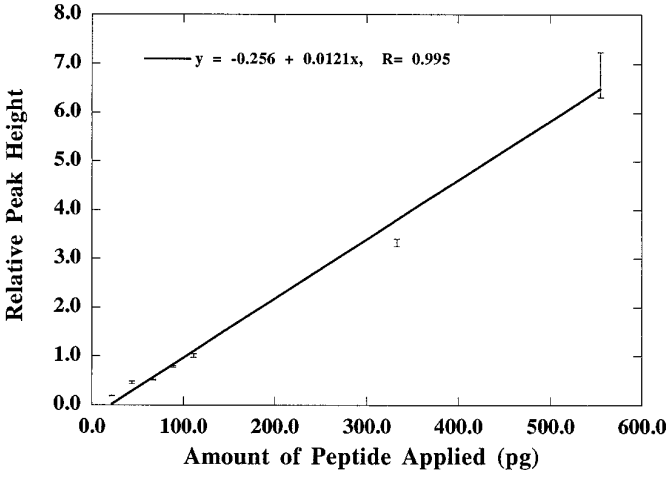

Fig. 3. Linear curve fit for the calibration of the mass spectrometric system with various amounts of E-2078 in 0.05% TFA. Error bars represent the S.E.M. of triplicate measurements for each standard solution. Relative peak height is the ratio of peak height of E-2078 to that of the internal standard, Dyn A (1–8).

in vivo in rhesus monkey blood. Detection of E-2078 in CSF after i.v. injection indicated that E-2078 had crossed the blood-brain barrier. Based on these studies, E-2078 is a suitable candidate for further pharmacological and neurobiological studies, as well as potentially a therapeutic agent both as analgesic and medicate for the treatment for specific addictions.

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