lected at intervals of 3 to 4 s. We performed bridge balance digitally off line using hyperpolarizing pulses of 50 to 100 pA preceding each trial. We detected spike times using thresholding of the first or second time derivative of the voltage. For all data collection and analysis we used Sun workstations with custom software written in C++ based on NEURON [M. Hines, in *Neural Systems: Analysis and Modeling*, F. H. Eeckman, Ed. (Kluwer, Boston, 1993), pp. 127–136].

13. G. J. Stuart and B. Sakmann, *Nature* **367**, 69 (1994).

· 14. The arrival of many uncorrelated excitatory and inhibitory synaptic events will deliver a total current to a neuron that may be treated approximately as shot noise [S. Rice, in *Selected Papers on Noise and Stochastic Processes*, N. Wax, Ed. (Dover, New York, 1954), pp. 133–294]. The event rates and their amplitude waveforms determine the mean, variance, and frequency spectrum of the net current. Accordingly, the stimuli used were realizations of Gaussian white noise with chosen mean (μ_s) and SD (σ_s) of fluctuations. Convolution with the function $f(t) = t \exp(-t/\tau_s)$ gave low-pass filtering with a time constant τ_s, as could be expected from synaptic time courses and dendritic filtering. Unless otherwise noted, τ_s was 3 ms. The range of σ_s investigated, 0 to 100 pA, produced voltage transients up to about 25 mV peak to peak.

15. Z. F. Mainen and T. J. Sejnowski, data not shown.

16. There was no systematic relation between μ_s and reliability over the range of values investigated (50 to 300 pA producing a firing rate of 4 to 32 Hz), although in some cells reliability did increase or decrease with μ_s.

17. The reverse correlation reported is similar to the first-order response kernel of the neuron [P. Z. Marmarelis and V. Z. Marmarelis, *Analysis of Physiological Systems: The White Noise Approach* (Plenum, New York, 1978)].

18. Z. F. Mainen and T. J. Sejnowski, unpublished observations.

19. N. A. Hessler, A. M. Shirke, R. Malinow, *Nature* **366**, 569 (1993); C. Rosenmund, J. D. Clements, G. L. Westbrook, *Science* **262**, 754 (1993); N. Otmakhov, A. M. Shirke, R. Malinow, *Neuron* **10**, 1101 (1993); C. Allen and C. F. Stevens, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10380 (1994); A. M. Thomson, J. Deuchars, D. C. West, *J. Neurophysiol.* **70**, 2354 (1993).

20. For example, bursts of action potentials may significantly increase the reliability (release probability) of a synapse through the mechanism of paired-pulse facilitation [R. S. Zucker, *Annu. Rev. Neurosci.* **12**, 13 (1989)].

21. J. H. R. Maunsell and J. R. Gibson, *J. Neurophysiol.* **68**, 1332 (1992); R. J. Snowden, S. Treue, R. A. Andersen, *Exp. Brain Res.* **88**, 389 (1992); J. R. Phillips, R. S. Johansson, K. O. Johnson, *J. Neurosci.* **12**, 827 (1992); W. Bair, C. Koch, W. T. Newsome, K. H. Britten, *Soc. Neurosci. Abstr.* **20**, 1229 (1994).

22. C. E. Carr and M. Konishi, *J. Neurosci.* **10**, 3227 (1990); N. Suga, J. F. Olsen, J. A. Butman, *Cold Spring Harbor Symp. Quant. Biol.* **55**, 585 (1990).

23. We are grateful to T. Zador, C. F. Stevens, C. Koch, and W. Bair for insightful comments and discussion. Z.F.M. is a Howard Hughes Medical Institute predoctoral fellow.

2 November 1994; accepted 24 March 1995

Chemical Characterization of a Family of Brain Lipids That Induce Sleep

Benjamin F. Cravatt, Oscar Prospero-Garcia, Gary Siuzdak, Norton B. Gilula, Steven J. Henriksen, Dale L. Boger,* Richard A. Lerner*

A molecule isolated from the cerebrospinal fluid of sleep-deprived cats has been chemically characterized and identified as *cis*-9,10-octadecenoamide. Other fatty acid primary amides in addition to *cis*-9,10-octadecenoamide were identified as natural constituents of the cerebrospinal fluid of cat, rat, and human, indicating that these compounds compose a distinct family of brain lipids. Synthetic *cis*-9,10-octadecenoamide induced physiological sleep when injected into rats. Together, these results suggest that fatty acid primary amides may represent a previously unrecognized class of biological signaling molecules.

The pursuit of endogenous sleep-inducing substances has been the focus of an extensive, complicated body of research (*1*). Several compounds, including delta-sleep-inducing-peptide (*2*) and prostaglandin PGD$_2$ (*3*), have been suggested to play a role in sleep induction, and yet, the molecular mechanisms of this physiological process remain largely unknown. We analyzed the cerebrospinal fluid of cats in search of com-

B. F. Cravatt, G. Siuzdak, D. L. Boger, R. A. Lerner, Department of Chemistry, Scripps Research Institute, La Jolla, CA 92307, USA.
O. Prospero-Garcia and S. J. Henriksen, Department of Neuropharmacology, Scripps Research Institute, La Jolla, CA 92307, USA.
N. B. Gilula, Department of Cell Biology, Scripps Research Institute, La Jolla, CA 92307, USA.

*To whom correspondence should be addressed.

pounds that accumulated during sleep deprivation. A molecule with the chemical formula $C_{18}H_{35}NO$ was isolated from the cerebrospinal fluid of sleep-deprived cats (*4*). The compound's structural features, two degrees of unsaturation, a long alkyl chain, and a nitrogen substituent capable of primary fragmentation as ammonia, were most compatible with either a nonconjugated diene in which a primary amine was allylic (*4*) or a monounsaturated alkane chain terminating in a primary amide (*5*).

Initial electrospray mass analysis of the natural compound revealed mass peaks of m/z 282 ([M + H]$^+$), 304 ([M + Na]$^+$), 320 ([M + K]$^+$), and 564 ([2M + H]$^+$), indicating that the molecular mass of the compound was 281 daltons (*4*). High-reso-

lution fast atom bombardment–mass spectrometry (FAB-MS) analysis indicated that the exact mass measurement of the [M + Na]$^+$ ion was m/z 304.2614 $\pm$ 0.0006 daltons. This measurement allowed for the determination of elemental composition and a best fit for the molecular formula $C_{18}H_{35}NO$, which has a calculated [M + Na]$^+$ m/z of 304.2616 daltons. Tandem mass spectrometry analysis (MS-MS or MS^2) revealed a distinctive fragmentation pattern in the low molecular mass range consistent with other long chain alkanes (Fig. 1A). Sequential neutral loss of 17 and 35 mass units from the parent ion indicated the loss of ammonia followed by the loss of water. Additional MS^3 experiments were performed on the daughter ions of m/z 265 and 247 (*4*).

Such MS^2 and MS^3 analyses were also performed on various synthetic candidate structures (*6*), and although several products gave spectra quite similar to those of the natural compound, only the fragmentation patterns generated from monounsaturated fatty acid amides, such as *cis*-9,10-octadecenoamide (Fig. 1B), matched exactly those of the endogenous lipid. Of interest was the neutral loss of 17 mass units from the parent ion of *cis*-9,10-octadecenoamide, indicating that the molecule first fragments at the carbon-nitrogen bond of its terminal amide group to release ammonia. Mass analysis also identified a compound from the cerebrospinal fluid of human and rat with the molecular formula $C_{22}H_{43}NO$ with MS^2 and MS^3 fragmentation patterns indistinguishable from those of synthetic *cis*-13,14-docosenoamide (Fig. 1, C and D) (*7*).

Cis-9,10-octadecenoamide and the C_{18} natural lipid exhibited identical elution properties on thin-layer chromatography (TLC) (*8*) and gas chromatography–mass spectrometry (GC-MS) (*9*). However, these techniques proved insensitive to the position and configuration of the olefin of closely related synthetic fatty acid amides, and the *cis*-8,9- (Fig. 2, 3), *cis*-9,10- (1), *cis*-11,12- (4), and *trans*-9,10- (2) octadecenoamides were not distinguishable from the natural compound by TLC and GC (*10*). Through infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and chemical degradation procedures, the exact structure of the endogenous lipid, including the position and configuration of its olefin, was unambiguously determined. The position of the double bond along the alkyl chain of the natural compound was determined by ozonolysis (*11*). GC-MS analysis of the ozonolysis reaction mixture derived from the natural lipid revealed nonyl aldehyde as the only CH$_3$-terminal aldehyde present. Nonyl aldehyde corresponds to an olefin located at the 9,10 position of the C_{18} fatty acid primary amide.

Fig. 1. Electrospray ionization tandem mass spectral data obtained for the natural compounds isolated from cerebrospinal fluid of sleep-deprived cats and their synthetic versions. Spectra in (**A**) and (**C**) represent the fragmentation data obtained for the natural C_{18} and C_{22} agents, respectively. Spectra in (**B**) and (**D**) represent the fragmentation data obtained for synthetic cis-9,10-octadecenoamide and cis-13,14-docosenoamide, respectively. The electrospray experiments were performed with an API III Perkin-Elmer SCIEX triple-quadrupole mass spectrometer.
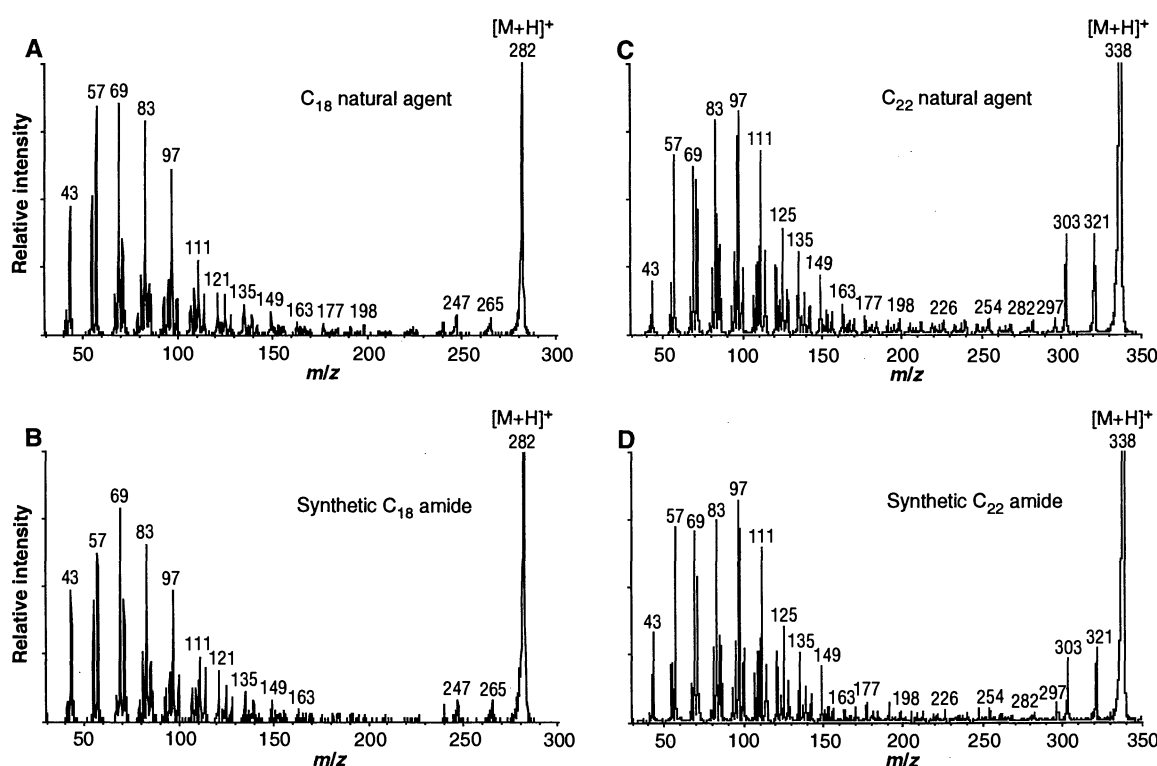
The IR spectrum of the natural compound (neat) exhibited absorbances at 3354 and 3320 (amide N–H stretches), 2923 and 2851 (alkane stretches), 1656 and 1630 (amide I and II bands), 1466, and 1410 cm^{-1}. Although cis-9,10-octadecenoamide gave a Fourier transform IR spectrum identical to that of the natural agent, a single characteristic difference was observed in the IR spectra of the endogenous lipid and trans-9,10-octadecenoamide: The trans isomer exhibited an additional strong absorption peak at 960 cm^{-1}. Neither the natural compound nor cis-9,10-octadecenoamide exhibited this IR absorption band characteristic of disubstituted trans alkenes (12).

Approximately 300 μg of the endogenous lipid (13) was used in NMR analysis. The ^{1}H NMR spectrum (CD_3OD, 400 MHz) exhibited the following peaks: δ 5.24 (multiplet, 2H, olefinic protons), δ 2.09 [triplet, 2H, $H_2NC(O)CH_2$], δ 1.93 (multiplet, 4H, allylic protons), δ 1.50 [multiplet, 2H, $H_2NC(O)CH_2CH_2$], δ 1.50 to 1.23 (multiplet, alkyl methylene protons), and δ 0.805 (triplet, 3H, CH_3). When compared with the ^{1}H NMR spectra of trans- and cis-9,10-octadecenoamide, the natural compound and cis-9,10-octadecenoamide were identical and definitively different from trans-9,10-octadecenoamide (Fig. 3). Samples of the natural lipid and cis-9,10-octadecenoamide were distinguishable from the trans isomer in both the olefinic and allylic regions of the ^{1}H NMR spectrum. Whereas the olefinic protons of the trans isomer reside at δ 5.28 in CD_3OD, the olefinic

protons of the natural compound and the cis isomer are shifted slightly upfield to δ 5.24. In the allylic region of the NMR spectrum, both the natural compound and the synthetic cis isomer have a four-proton peak ranging from δ 1.94 to 1.91, whereas the allylic protons of the trans isomer are observed at δ 1.88 to 1.86. Trace amounts of the trans fatty acid amide do appear to be present as well in the natural sample, indicating perhaps that this agent is also an endogenous constituent of the brain. Thus, through the use of MS, GC, TLC, IR, NMR, and ozonolysis the structure of the unknown natural lipid was determined to be cis-9,10-octadecenoamide (Fig. 2, 1).
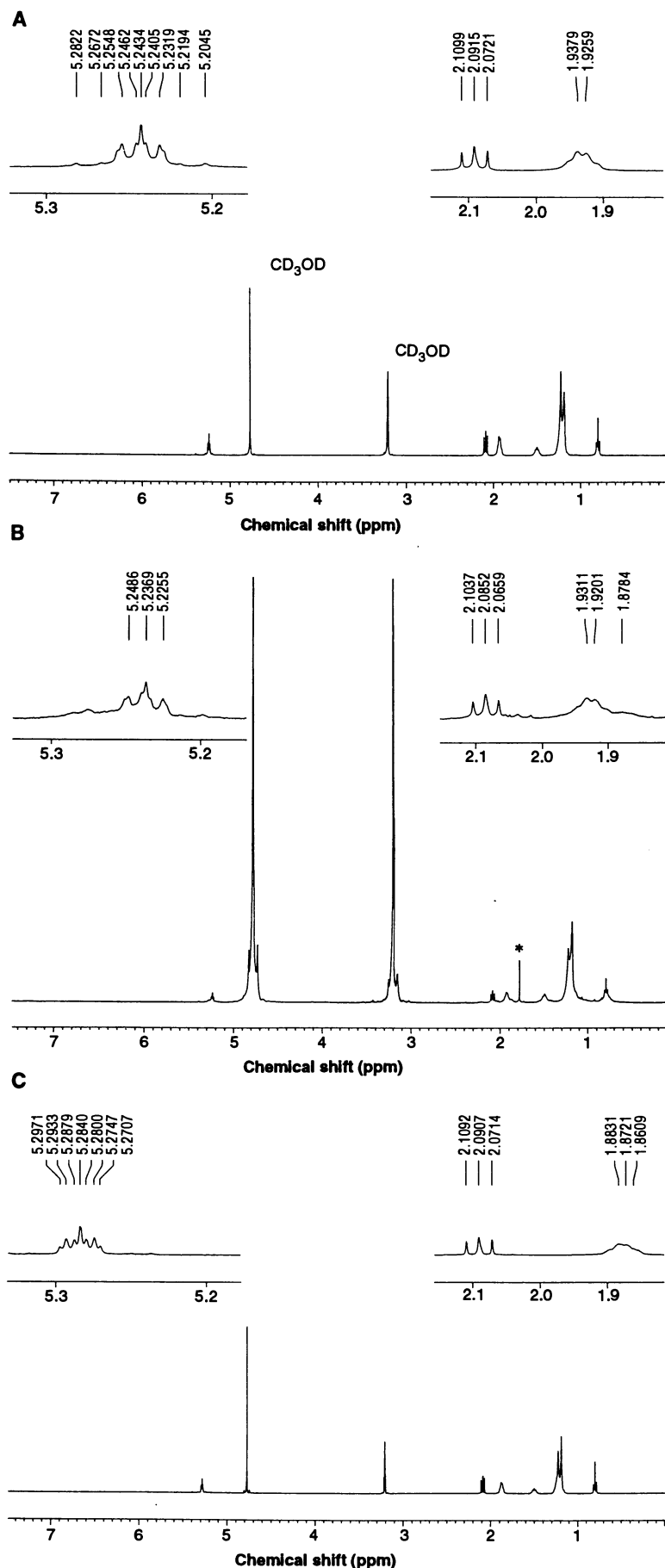
Synthetic cis-9,10-octadecenoamide was injected (intraperitoneal) into rats [1 ($n = 2$), 2 ($n = 2$), 5 ($n = 7$), 10 ($n = 10$), 20 ($n = 2$), and 50 ($n = 2$) mg, where n is the number of rats tested] during a reversed dark period (12 hours of light:12 hours of dark) 2 hours after the lights cycled off. The lower doses (1 and 2 mg) produced no overt effect on spontaneous behavior. However, with doses of 5 mg and above there was an induction of long-lasting motor quiescence associated with closed eyes and sedated behavior characteristic of normal sleep (14). As in normal sleep, the rats still responded to auditory stimuli with an orienting reflex and sustained attention toward the source of stimulation. The latency to behavioral sedation was about 4 min, and the subjects were normally active again after 1 hour (5 mg), 2 hours (10 mg), or 2.5 hours (20 and 50 mg). An intraventricular injection of 2.8

μg (10 nmol, $n = 2$) of synthetic cis-9,10-octadecenoamide also induced electrophysiologically monitored sleep, indicating that the agent acts directly in the brain at a dose comparable with other known effector molecules (1).

We have compared the sleep-inducing properties of cis-9,10-octadecenoamide to those of the vehicle and synthetic analogs to estimate the structural basis for the biological effect. Neither the vehicle (5% ethanol in saline solution) nor oleic acid (5) showed any overt behavioral effect or modified the spontaneous sleep-wake cycle. Trans-9,10-octadecenoamide (2) exhibited similar pharmacological effects to
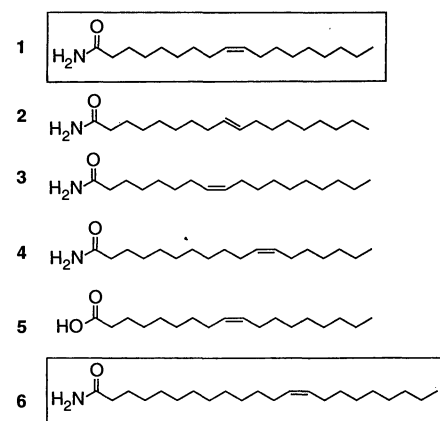

Fig. 2. Structures of natural agent cis-9,10-octadecenoamide (**1**) and related analogs (**2** to **6**). Compound **6** is the proposed structure of naturally occurring C_{22} fatty acid primary amide.

Fig. 3. ^{1}H NMR spectra of (**A**) synthetic *cis*-9,10-octadecenoamide, (**B**) the natural compound, and (**C**) synthetic *trans*-9,10-octadecenoamide. Expanded are the regions of the spectra that distinguish between the cis and trans isomers (olefinic protons from δ 5.3 to 5.2 and allylic protons from δ 2.0 to 1.8). These regions identify the natural compound as *cis*-9,10-octadecenoamide. The asterisk in (B) indicates an impurity.

its cis counterpart, but it was much less potent, as measured by the comparatively shorter duration time for its sleep-inducing properties (at 10 mg per rat, the biological effect lasted 1 hour for the trans isomer and 2 hours for the cis isomer). Moving the olefin either direction along the alkyl chain [to the 8,9 (**3**) or 11,12 (**4**) positions] or extending the alkyl chain length to 22 carbons (**6**) substantially reduced both the degree and duration of the effect, and although the mobility of the rats still decreased, their eyes remained open and their alertness was only slightly affected.

The biological concentration of neuroactive signaling molecules should expectably adhere to tight regulation. To evaluate this possibility, we searched for an enzymatic activity capable of degrading the putative effector molecule, *cis*-9,10-octadecenoamide (*15*). Rapid conversion of ^{14}C-labeled *cis*-9,10-octadecenoamide to oleic acid by rat brain membrane fractions (*16*) was observed by TLC (Fig. 4). The enzymatic activity was unaffected by 5 mM EDTA but was completely inhibited by 1 mM phenylmethylsulfonyl fluoride
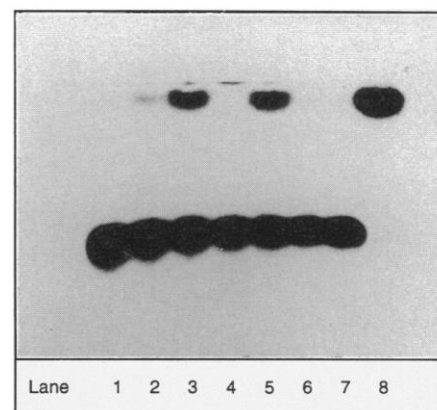
Fig. 4. *Cis*-9,10-octadecenoamide hydrolysis to oleic acid by rat brain membrane fractions. TLC (SiO$_2$ sorbent and a solvent of 55% ethyl acetate in hexanes) analysis: Lane 1, *cis*-9,10-octadecenoamide standard; lane 2, *cis*-9,10-octadecenoamide with rat brain soluble fraction; lane 3, *cis*-9,10-octadecenoamide with rat brain membrane fraction; lane 4, *cis*-9,10-octadecenoamide with rat brain membrane fraction plus 1 mM PMSF; lane 5, *cis*-9,10-octadecenoamide with rat brain membrane fraction plus 5 mM EDTA; lane 6, *cis*-9,10-octadecenoamide with rat pancreatic microsomes; lane 7, *cis*-9,10-octadecenoamide with proteinase K (200 µg); and lane 8, oleic acid standard.

(PMSF). Only trace amide hydrolysis activity was observed with rat brain soluble fractions, whereas rat pancreatic microsomes and proteinase K showed no significant capacity to hydrolyze cis-9,10-octadecenoamide to oleic acid.

The fatty acid amides we have studied belong to a family of simple molecules in which a great deal of diversity may be generated by simply varying the length of the alkane chain and the position, the stereochemistry, and the number of its olefins. Other neuroactive signaling molecules with amide modifications at their COOH-termini have been reported, from carboxamide terminal peptides (17) to arachidonylethanolamide (18). Perhaps cis-9,10-octadecenoamide is a member of a class of biological effector molecules in which simple variations of a core chemical structure have distinct physiological consequences. Alternatively, given the enzymatic hydrolysis of cis-9,10-octadecenoamide by rat brain membranes, we cannot exclude the possibility that the liberated ammonia or other modifications of the agent may be involved in the effector function.

REFERENCES AND NOTES

1. S. Inoue, Biology of Sleep Substances (CRC, Boca Raton, FL, 1989).
2. G. A. Schoenenberger, Eur. Neurol. 23, 321 (1984).
3. R. Ueno et al., Biochem. Biophys. Res. Commun. 109, 576 (1982).
4. R. A. Lerner et al., Proc. Natl. Acad. Sci. U.S.A. 91, 9505 (1994).
5. E. S. Arafat et al., Life Sci. 45, 1679 (1989); K. Wakamatsu et al., Biochem. Biophys. Res. Commun. 168, 423 (1990); M. K. Jain et al., J. Med. Chem. 35, 3584 (1992).
6. α-Amino ketones (4) were readily distinguishable from the natural agent by fragmentation pattern analysis. $\Delta^{3,4}$-1-Hydroxy-2-aminooctadecadienes (4) exhibited nearly identical fragmentations to the natural lipid, only differing in the relative intensities of lower molecular weight fragments in MS^2 experiments.
7. The natural compound $C_{22}H_{43}NO$ and synthetic cis-13,14-docosenoamide also exhibited identical elution properties on TLC, and ozonolysis experiments indicated that the olefin of the C_{22} natural agent is in the 13,14 position.
8. SiO$_2$ and a solvent of 75% ethyl acetate in hexanes was used for TLC, and the compounds had an R_f (ratio of the distance traveled by a compound to that traveled by the solvent) of 0.3.
9. GC-MS analyses were carried out on a 5890 Hewlett-Packard gas chromatograph with a 5971A Hewlett-Packard mass-selective detector. Separations were performed on a DB-5 (0.25-μm film) capillary column that was 30 mm in length and had an internal diameter of 0.25 mm.
10. Cis-9,10-octadecenoamide, cis-11,12-octadecenoamide, and trans-9,10-octadecenoamide were prepared from their respective acids as follows: cis-9,10-octadecenoic acid (oleic acid) in CH_2Cl_2 (0.2 M) at 0°C was treated with oxalyl chloride [3 equivalents (eq)] and stirred at 25°C for 4 hours. Removal of the solvent followed by treatment with saturated aqueous NH$_4$OH at 0°C gave cis-9,10-octadecenoamide, which was purified by silica gel column chromatography [a gradient of ethyl acetate (40 to 75%) in hexanes was used for elution]. Cis-8,9-octadecenoamide was synthesized from the phosphonium salt of methyl 8-bromooctanoate [S. Genard and H. Patin, Bull. Soc. Chim. Fr. 128, 397 (1991)]. The phosphonium salt was dissolved in tetrahydrofuran at 25°C and treated with potassium bis(trimethylsilyl)amide (1.1 eq). After it was stirred at reflux for 1 hour, the solution was cooled to −78°C and decanal (1.5 eq) was added. The mixture was allowed to warm to 25°C and stirred for 30 min to afford methyl cis-8,9-octadecenoate. Methyl cis-8,9-octadecenoate was hydrolyzed to afford cis-8,9-octadecenoic acid with LiOH (3 eq) in THF-CH$_3$OH-H$_2$O (3:1:1). Conversion of cis-8,9-octadecenoic acid to cis-8,9-octadecenoamide was conducted as described above.
11. O. S. Privett, Prog. Chem. Fats Other Lipids 9, 91 (1966).
12. W. W. Christie, Lipid Analysis (Pergamon, Oxford, 1973), pp. 128.
13. After isolation by high-performance liquid chromatography (HPLC) of the natural compound as described previously (4), the HPLC fractions were combined, concentrated, and resuspended in the minimum volume of CH_2Cl_2. Silica gel chromatography (a 40 to 75% gradient of ethyl acetate in hexanes) of the crude product afforded approximately 300 μg of the natural compound. Residual impurities including grease at δ 1.2 account for the variation in the ^{1}H NMR peak intensities in this region when compared with synthetic cis-9,10-octadecenoamide.
14. Cis-9,10-octadecenoamide increased the total time of slow wave sleep (SWS) as well as the mean duration of the individual SWS periods when compared with the vehicle and oleic acid controls [standard electrographic methodologies; see O. Prospero-Garcia, J. R. Criado, S. J. Henriksen, Pharmacol. Biochem. Behav. 49 413, (1994)]. These increases were at the expense of waking.
15. The following protocol was used to assay the cis-9,10-octadecenoamide hydrolysis activity. To 120 μl of 125 mM tris-HCl, pH 9.0, was added successively 70 μl of protein fraction (protein concentrations of 1 mg/ml for brain membrane fraction, 2 mg/ml for brain soluble fraction, and 5 mg/ml for pancreatic microsomal preparation), 6 μl of ethanol, and 4 μl of ^{14}C-cis-9,10-octadecenoamide (in ethanol, 0.25 μCi/μl). Each reaction mixture was incubated for 4 hours at 37°C and then partitioned between ethyl acetate and 0.07 M HCl. The ethyl acetate layer was evaporated to dryness and the remaining residue was dissolved in 15 μl of ethanol. TLC fractions were taken from this ethanol stock. TLC plates were treated with EN^{3}HANCE spray and developed at −78°C for 2 hours.
16. Brain membrane fractions were prepared according to W. A. Devane and J. Axelrod [Proc. Natl. Acad. Sci. U.S.A. 91, 6698 (1994)].
17. B. A. Eipper, D. A. Stoffers, R. E. Mains, Annu. Rev. Neurosci. 15, 57 (1992).
18. W. A. Devane et al., Science 258, 1946 (1992).
19. We thank L. Bibbs for HPLC assistance, T. Ramsey for help with Fig. 3, G. Klier for help with Fig. 4, R. Safarik for guidance on tissue preparations, and M. Falk for the rat pancreatic microsomes. Supported by the Lucille P. Markey Charitable Trust, NIH Shared Instrumentation grant 1 S10 RR07273-01, and NSF (predoctoral fellowship, B.F.C.).