Chemical Characterization of a Family of Brain Lipids That Induce Sleep

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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 PGD2 (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 compounds that accumulated during sleep deprivation. A molecule with the chemical formula C16H31NO 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-resolution fast atom bombardment–mass spectrometry (FAB-MS) analysis indicated that the exact mass measurement of the [M + Na]+ ion was m/z 304.2614 ± 0.0006 daltons. This measurement allowed for the determination of elemental composition and a best fit for the molecular formula C16H31NO, which has a calculated [M + Na]+ m/z of 304.2616 daltons. Tandem mass spectrometry analysis (MS-MS or MS2) 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 MS3 experiments were performed on the daughter ions of m/z 265 and 247 (4).

Such MS2 and MS3 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 mono-unsaturated 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 C16H31NO with MS2 and MS3 fragmentation patterns indistinguishable from those of synthetic cis-13,14-docosenoamide (Fig. 1, C and D) (7).

Cis-9,10-octadecenoamide and the C16 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 CH1-terminal aldehyde present. Nonyl aldehyde corresponds to an olefin located at the 9,10 position of the C16 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 C₁₈ and C₂₂ 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 absorptions 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 ¹H NMR spectrum (CD₃OD, 400 MHz) exhibited the following peaks: δ 5.24 (multiplet, 2H, olefinic protons), δ 2.09 (triplet, 2H, H₂NC(O)CH₂), δ 1.93 (multiplet, 4H, allylic protons), δ 1.50 (multiplet, 2H, H₂NC(O)CH₂CH₂), δ 1.50 to 1.23 (multiplet, alkyl methylene protons), and δ 0.805 (triplet, 3H, CH₃). When compared with the ¹H 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-octadecenoic acid (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 ¹H NMR spectrum. Whereas the olefinic protons of the trans isomer reside at δ 5.28 in CD₃OD, the olefinic protons of the natural compound and the cis isomer are shifted slightly upward 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 coenzymology 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) μg, 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 a clear 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 C₂₂ fatty acid primary amide.

1. \[\text{cis-9,10-octadecenoamide} \]
2. \[\text{trans-9,10-octadecenoic acid} \]
3. \[\text{cis-13,14-docosenoamide} \]
4. \[\text{trans-13,14-docosenoic acid} \]
5. \[\text{cis-10,11-docosenoamide} \]
6. \[\text{trans-10,11-docosenoic acid} \]
Fig. 3. 'H NMR spectra of (A) synthetic cis-9,10-octadecenoic acid, (B) the natural compound, and (C) synthetic trans-9,10-octade-
cenoic acid. Expanded are the regions of the spectra that distinguish
between the cis and trans isomers (olefinic protons from 6 5.3 to 5.2
and allylic protons from 6 2.0 to 1.8). These regions identify the
natural compound as cis-9,10-octadecenoic acid. The asterisk
in (E) 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 car-
bons (6) substantially reduced both the de-
gree 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 neuro-
active signaling molecules should expect-
ably adhere to tight regulation. To evalu-
ate this possibility, we searched for an
enzymatic activity capable of degrading
the putative effector molecule, cis-9,10-
octadecenoic acid (15). Rapid conversion of
14C-labeled cis-9,10-octadecenoic acid
to oleic acid by rat brain membrane frac-
tions (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-octadecenoic acid hydrolysis to
oleic acid by rat brain membrane fractions. TLC
(SiO2 sorbent and a solvent of 55% ethyl acetate
in hexanes) analysis: Lane 1, cis-9,10-octade-
cenoic acid standard; lane 2, cis-9,10-octade-
cenoic acid with rat brain soluble fraction; lane 3,
cis-9,10-octadecenoic acid with rat brain mem-
brane fraction; lane 4, cis-9,10-octadeceno-
amic acid with rat brain membrane fraction plus
1 mM PMSF; lane 5, cis-9,10-octadecenoic acid
with rat brain membrane fraction plus 5 mM
EDTA; lane 6, cis-9,10-octadecenoic acid with rat
pancreatic micromes; lane 7, cis-9,10-octade-
cenoic acid 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 protease 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 neurotransmitter signaling molecules with amide modifications at their COOH-termini have been reported, from carboxamide terminal peptides (17) to arachidonylthanolamide (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

6. α-Amino ketones (4) were readily distinguishable from the natural agent by fragmentation pattern analysis. Δ5,6-1-Hydroxy-2-aminoocitaredenoids (4) exhibited nearly identical fragmentations to the natural lipid, only differing in the relative intensities of lower molecular weight fragments in MSF experiments.
7. The natural compound C19H26NO and synthetic cis-13,14-docosanoamide also exhibited identical elution properties on TLC, and ozonolysis experiments indicated that the olefin of the C19 natural agent is in the 13,14 position.
8. SOCl2 and a solvent of 75% ethyl acetate in hexanes was used for TLC, and the compounds had an Rf (ratio of the distance traveled by a compound to that traveled by the solvent) of 0.5.
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-octadecenoic acid, cis-11,12-octadecenoic acid, and trans-9,10-octadecenoic acid were prepared from their respective acids as follows: cis-9,10-octadecanoic acid (oleic acid) in CH2Cl2 (0.2 M) at 0°C was treated with oxalic chloride (3 equivalents eq) and stirred at 25°C for 4 hours. Removal of the solvent followed by treatment with saturated aqueous NH4OH at 0°C gave cis-9,10-octadecenoic acid, which was purified by silica gel column chromatography (a gradient of ethyl acetate 40 to 75% in hexanes was used for elution). cis-9,10-octadecenoic acid was synthesized from the phosphonium salt of metheyl-bromoacetanate (S. Gerhard and H. Pattn, J. Org. Chem. 39, 597 (1974)). The phosphonium salt was dissolved in tetrahydrofuran at 25°C and treated with potassium tert-butoxylamine (1.1 eq). After it was stirred at reflux for 1 hour, the solution was cooled to -78°C and decanl (1.5 eq) was added. The mixture was allowed to warm to 25°C and stirred for 30 minutes to afford methyl cis-9,10-octadecenoate. Methyl cis-9,10-octadecenoate was hydrolyzed to afford cis-9,10-octadecenoic acid with LiOH (5 eq) in THF·CH3OH·H2O (6:1:1). Conversion of cis-9,10-octadecenoic acid to cis-9,10-octadecenoamide was conducted as described above.
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 CH3Cl. Cis-12-gel chromatography (5 to 75% gradient of ethyl acetate in hexanes) of the crude product afforded approximately 300 μg of the natural compound. Residual impurities included unprocessed oil at 2% account for the variation in the 1H NMR peak intensities in this region when compared with synthetic cis-9,10-octadecenoic acid.
14. cis-9,10-octadecenoic acid 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 saline acid controls (standard electrophysiological methodologies; see also. Prosero-Garcia, J. R. Criado, S. J. Herrinksen, Pharmacol. Biochem. Behav. 49, 143, (1994)). These increases were at the expense of waking.
15. The following protocol was used to assay the cis-9,10-octadecenoic acid 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 9C-cis-9,10-octadecanoic acid (in ethanol, 0.25 μg/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 12 μl of ethanol. TLC fractions were taken from this ethanolic stock. TLC plates were treated with ENHANCE spray and developed at -78°C for 2 hours.
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