Chapter 15

# New Directions in the Analysis of Brain Substances Related to Sleep and Wakefulness

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## Contents



## I. A Brief History of CSF Characterization

Between 1916 and 1960 cerebrospinal fluid (CSF) was believed to be little more than an ideal physiological saline solution, unidentifiable by any known histochemical method.[1] In the 1970s, characterization of CSF began in earnest and as the technology for its analysis developed, much was learned about the cation, anion, and nonelectrolyte (specifically urea and glucose) content of CSF. The primary

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species identified therein were $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $Cl^-$, $HCO_3^-$, $^{82}Br^-$, $^{131}I^-$, $CNS^-$, glucose, and urea. During this period it was also hypothesized that CSF had additional complexity relating to its interactions with blood plasma across the blood–brain barrier.

In the 1980s, significant discoveries within CSF included sphingolipids, brain-specific proteins, nucleic acids, transfer RNAs, and amino acids. Beyond discovering the mere presence of these molecules, their detailed characterization was possible through advances in analytical technology, namely gas chromatography mass spectrometry, DNA sequencing, and amino acid sequencing. Substances which had been previously overlooked, like myelin basic protein, S-100 protein, NP (ribonucleoprotein), 14-3-2 protein, antigen-$\alpha$, and $\alpha_2$-glycoprotein, could now be completely characterized.

## II. Gaps in Existing Knowledge

The increased knowledge of the content of cerebrospinal fluid has brought with it a vast array of new determinants for the presence and course of diseases. By gaining a better understanding of CSF, physicians and researchers alike have been able to better detect and monitor treatment effectiveness for diseases such as Alzheimer's, AIDS-related neurological disease, muscular sclerosis, and tuberculosis meningoencephalitis. Unfortunately, it is clear that the current reference files for CSF are far from complete. While physicians are relying more and more on clinical chemistry laboratories for biochemical analysis of patients' CSF, the clinical value of many CSF analytes outside of glucose and protein determinations is often ambiguous. Thus, correlating existing analytes and identifying new analytes that correlate with specific physiological processes has immediate utility. Much of the current work on CSF is still directed toward finding the process or pathway of exchange and transport of the CSF from the brain to the locations in which its constituents are utilized. Still lacking is complete knowledge of its actual content and the relationship of these compounds with physiological processes. The goal in current research lies in identifying these molecules primarily through new analytical techniques.

## III. Filling the Gap

Our group has taken a purely chemical approach in its attempt to identify molecules of the central nervous system which vary according to various physiological stimuli. Of particular interest has been the identification of a compound or compounds correlating to sleep.[2] A driving force for these experiments was the realization that the sensitivity of modern analytical techniques could allow for a fresh look at CSF constituents.[3-5] Most notably, the sensitivity offered by mass spectrometry has provided a route by which picomole to femtomole amounts of a compound may be analyzed, whereby thermally labile biomolecules are less apt to fragment and decompose than they were with earlier mass analysis techniques such as electron ioniza-

tion.[6] The goal of our experiments has been to use this technology to identify new molecules in CSF associated with the sleep–wake cycle.

## A. Mass Spectrometry

The attempt to identify sleep molecules in CSF requires an efficient method for screening its molecular content. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry both offer molecular weight information on these types of compounds. In fact, it has been the development of these mass analysis techniques, allowing higher levels of sensitivity, increased mass range, and better mass accuracy, along with new sampling methods, that have led to an increasing number of mass spectrometry-based applications.[6,7]

ESI and MALDI are fundamentally very different ionization techniques, yet they achieve essentially the same result — the generation of gas phase ions via nondestructive vaporization and ionization. In both techniques, ionization typically occurs through proton addition or proton abstraction to produce either $[M+H]^+$ or $[M-H]^-$ ions (where M is the molecule of interest). In matrix-assisted laser desorption/ionization, gas phase ions are generated by the laser vaporization of a solid matrix/analyte mixture in which the matrix (usually a small crystalline organic compound) strongly absorbs the laser radiation and acts as a receptacle for energy deposition.[8] This concentrated energy deposition results in the vaporization and ionization of both matrix and analyte ions containing very few charges. The relatively low number of charge states observed in MALDI makes the technique especially well suited for the analysis of multicomponent mixtures because individual components can be easily identified by the signal generated from their 1+ charge state. This ability to analyze heterogeneous samples has made it especially useful for looking at biological solutions. While not used extensively in this study, MALDI has been useful for the analysis of biopolymers in CSF,[9] and has become the method of choice for large biopolymer (>30 KDa) analysis. ESI, on the other hand, has become more important for analyzing smaller biomolecules, and was therefore our method of choice.

In ESI-MS, ions are formed directly from solution (usually an aqueous or aqueous/organic solvent system) by creating a fine spray of highly charged droplets in the presence of a strong electric field (Figure 15.1). Subsequent vaporization of these charged droplets results in the production of charged gaseous ions. The number of charges retained by an analyte can depend on such factors as the composition and pH of the electrosprayed solvent as well as the chemical nature of the sample. For large molecules, the ESI process typically gives rise to a series of multiply charged species for a given analyte. Because mass spectrometers measure the mass-to-charge ($m/z$) ratio, the resultant ESI mass spectrum contains multiple peaks corresponding to the different charged states (Figure 15.1).

An important feature of ESI-MS is its ability to directly analyze compounds from aqueous or aqueous/organic solutions, a feature that has established the technique as a convenient mass detector for liquid chromatography (LC). ESI also allows for MS analysis at relatively high LC flow rates (1.0 ml/min) and high mass accuracy

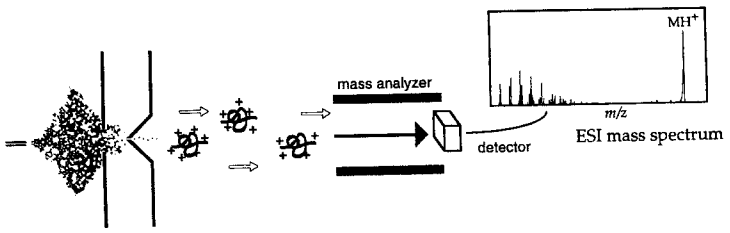



**FIGURE 15.1**
Schematic representation of electrospray ionization (ESI). ESI occurs via charged droplet evaporation, and the ionized species are directed through a mass analyzer which allows for differentiation and detection of the ions according to their mass-to-charge ratio (*m/z*).

(±0.01%), adding a new dimension to the capabilities of LC characterization. In fact, using ESI-MS as a detector for LC was one of its first obvious applications. Numerous reports have been published on this with special emphasis on small molecule, peptide, and protein analysis. The combination of LC and ESI-MS is excellent for routine and reproducible molecular weight determinations in a wide variety of compounds, whether they are positively (i.e., peptides and proteins) or negatively (i.e., oligonucleotides) charged (Figure 15.2). This is especially useful since the CSF contains both cationic and anionic compounds.

## B. Tandem Mass Spectrometry

While molecular weight information is of course useful in the preliminary stages of molecular characterization, it is also important to gather structural information. Tandem mass spectrometry, with its ability to induce fragmentation and perform successive mass spectrometry experiments on these fragment ions, is generally used to obtain this structural information (abbreviated $MS^n$ — where **n** refers to the number of generations of fragment ions being analyzed). The technique is illustrated in Scheme 15.1.

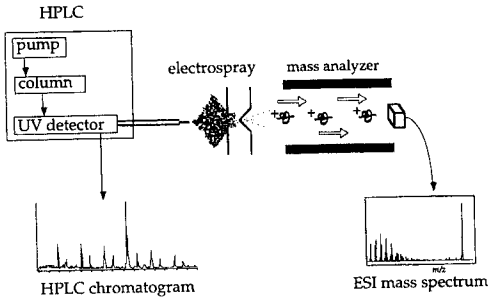
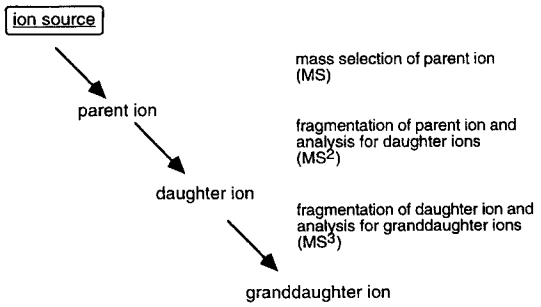



**FIGURE 15.2**
Interfacing liquid chromatography with electrospray ionization mass spectrometry.




**SCHEME 15.1**

Fragmentation is usually achieved by inducing ion/molecule collisions. This process, known as collision-induced dissociation (CID), is accomplished by selecting an ion of interest with the mass analyzer and introducing that ion into a collision cell. The selected ion will hit a collision gas, such as argon, which may result in fragmentation. The fragments can then be analyzed to obtain a daughter ion spectrum. ESI typically uses a triple quadrupole or an ion trap mass spectrometer with collision-induced dissociation to perform these analyses. A brief description of $MS^2$ experiments with a triple quadrupole mass analyzer is given in Figure 15.3.

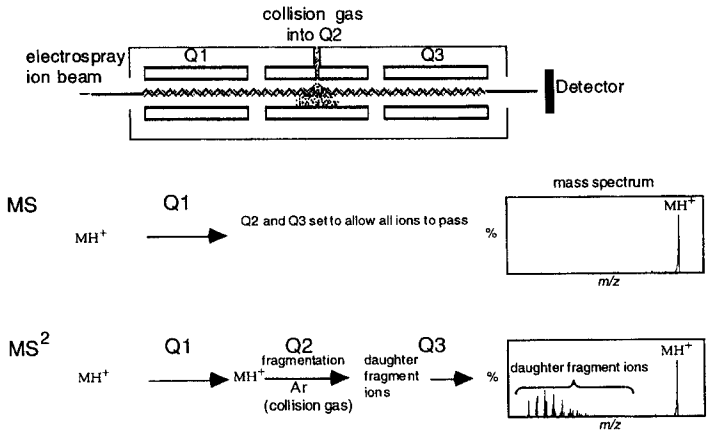



**FIGURE 15.3**

A triple quadrupole ESI mass spectrometer with ion selection and fragmentation capabilities. Each quadrupole has a separate function: The first quadrupole (Q1) is used to scan across a preset *m/z* range or to select an ion of interest; the second quadrupole (Q2), also known as the collision cell, transmits the ions while introducing a collision gas (argon) into the flight path of the ion selected by Q1; and the third quadrupole (Q3) serves to analyze the fragment ions generated in the collision cell (Q2). For an MS experiment Q1 scans over a selected *m/z* range and all the ions are observed. In the $MS^2$ experiment, the molecular ion $M^+$ can be selected by Q1, which results in its fragmentation at Q2. Analysis of the fragments occurs at Q3.

# IV. Applications to CSF Studies

In the sleep/CSF studies, fragmentation data were extremely helpful because preliminary analysis of the observed compounds allowed us to gain some characterization information. For example, Figure 15.3 illustrates a fragmentation pattern that allowed us to identify the 22 carbon lipid amide, euricamide. Although this particular compound has not exhibited any sleep-inducing properties in rats, its identification was interesting nonetheless and was one of many results obtained directly from our mass spectral analysis.

With the goal of identifying new molecules associated with the sleep–wake cycle, the sleep–wake states of feline subjects were examined. CSF analysis began with preparative liquid chromatography fraction collection in which UV data were used to determine any differences between the felines' CSF at various points in their sleep cycle. The LC analysis of the felines' CSF produced a peak which was present in sleep-deprived, but not in normal cats. An absorbance in the LC chromatogram was found to be particularly prominent in the CSF of cats that were kept awake for an extended period of time (18 hours). The fractions isolated by LC were then analyzed by electrospray mass spectrometry and electrospray tandem mass spectrometry. Even though the compound associated with this absorbance peak was only present in small amounts, partial characterization was initially obtained by performing electrospray tandem mass analysis and exact mass measurements.

## A. Identification

Electrospray mass analysis on the fractions associated with the differences in the chromatogram produced a significant ion at $MH^+$ *m/z* 282, while exact mass determination on the unknown compound using fast atom bombardment (FAB) mass analysis provided a molecular formula of $C_{18}H_{35}NO$. Collision-induced dissociation (CID) was used to perform $MS^2$ and $MS^3$ experiments on the ion at *m/z* 282, in which tandem mass analysis (Scheme 15.1) at *m/z* 282 revealed a distinct fragmentation pattern in the low molecular mass range, consistent with other long-chain alkanes. Neutral losses of 17 and 35 Da from the parent ion indicated a loss of ammonia followed by water. Performing additional $MS^3$ experiments on the daughter ions at *m/z* 265 and 247 revealed that the daughter ion at 265 fragmented to form the granddaughter ion at 247. This suggested that the ion at 247 was the result of sequential losses (loss of 17 Da [$NH_3$] followed by 18 Da [$H_2O$]), as opposed to a neutral loss independent of the daughter ion at 265. Additional deuterium exchange experiments were consistent with at least two protons on this molecule being exchangable. These results suggested that we had a fatty amide containing one point of unsaturation.

Chemical degradation techniques were first employed on synthetic fatty acid amides, identifying ozonolysis as conducive to the analysis of these agents. GCMS analysis of the ozonolysis reaction mixture derived from the natural lipid revealed nonyl aldehyde as the only C-terminal aldehyde present. Nonyl aldehyde corresponds to an olefin positioned seven methylenes away from the terminal methyl

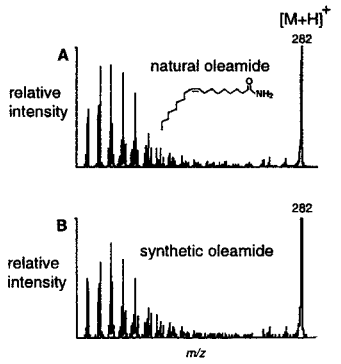



**FIGURE 15.4**
Electrospray ionization tandem mass spectra of natural oleamide found in CSF (A) and the synthetic version (B).

group of the alkyl chain, which in the case of a C18 fatty acid primary amide, is the 9,10 position.

On the basis of these experiments, compounds that best correlated to the data were synthesized and spectroscopically evaluated. The synthesis of fatty amides, combined with NMR and IR spectroscopic and mass spectral data of these compounds led to the unknown identification as *cis*-9,10-octadecenoamide or oleamide (Figure 15.4). A summary of the data appears in Table 15.1.

**Mass Spectrometry.** The mass spectrometry experiments were performed on an API III Perkin Elmer SCIEX triple-quadrupole mass spectrometer and a Fisons/VG ZAB-VSE high resolution magnetic sector mass spectrometer. The pneumatically assisted electrospray interface on the SCIEX was used for sample intro-

**TABLE 15.1**
**Summary of Data Generated on Oleamide**

| Data type | Summary of data |
|---|---|
| Cationization | Electrospray MS observation of *m/z* 304 $[M + Na]^+$ and *m/z* 320 $[M + K]^+$ confirms that M = 281. |
| Deuterium exchange | *m/z* = 285 $[M - 2H^+ + 3D^+]^+$, at least 2 exchangeable protons. |
| Exact mass | Observed 304.2614 Da |
| | Theoretical 304.2616 Da exact mass for $[C_{18}H_{35}NO + Na]^+$ |
| ESI-$MS^2$ | Fragment ion *m/z* 265 corresponding to $[MH - NH_3]^+$. |
| | Fragment ion *m/z* 247 corresponding to $[MH - NH_3 - H_2O]^+$. |
| | Lipid fragment ions. |
| ESI-$MS^3$ | Fragmentation of *m/z* 265 >> *m/z* 247 (sequential loss) suggests *m/z* 247 fragment is related to *m/z* 265 fragment ion. |
| | Lipid fragment ions. |
| NMR data | Confirms lipid portion of molecule and configuration of double bond. |
| Ozonolysis with GC/MS | Location of the double bond. |
| IR data | Identities unknown as *cis* isomer. |

duction with the potential of the interface sprayer maintained at 5.0 kV. A curtain gas of ultra pure nitrogen (1.0 liter/min.) between the interface plate and the sampling orifice was applied to aid desolvation of the charged droplets and to prevent particulate matter from entering the analyzer region. Samples were introduced through the interface at a rate of 4.0 μl/minute. The positive ions generated by the ion evaporation process entered the analyzer through an interface plate and a 100 μm orifice. The declustering potential was maintained between 50 and 250 V (typically 100 V) to control the collisional energy of the entering ions. A cryogenic pump was used to cool the surfaces within the spectrometer (14 to 18K) maintaining a working pressure of $2 \times 10^{-5}$ Torr and a sealed pressure of $8 \times 10^{-8}$ Torr in the analyzer region. The Fisons/VG ZAB-VSE instrument was used to perform the exact mass determination on the unknown compound by fast atom bombardment (FAB) analysis; a resolution of 10,000 (10% valley definition) was obtained to perform an exact mass measurement to within 0.7 parts per million.

**Collision-Induced Dissociation (CID).** The CID experiments were performed on the API III Perkin Elmer SCIEX triple-quadrupole mass spectrometer with ultra pure argon (>99.99% purity) as a collision gas. The positive ion MS/MS data was acquired by mass selecting the precursor ion with the first quadrupole, after which collisions with argon (target thickness of $3 \times 10^{14}$ atom/cm$^2$) in the second quadrupole produced dissociation. The third quadrupole mass-analyzed the resultant daughter ions. Collision energies of 80 eV were maintained in these experiments. CID spectra shown were the result of averaging from 50 to 100 scans depending on the number of scans necessary to obtain a signal-to-noise greater than or equal to 50. MS/MS/MS ($MS^3$) data were also obtained by mass selecting a daughter ion generated at the orifice with the first quadrupole mass analyzer. Collisions with argon (target thickness of $3 \times 10^{14}$ atom/cm$^2$) in the second quadrupole collision cell produced further dissociation, and the resultant granddaughter ions were analyzed with the third quadrupole mass analyzer.

## B. Characterizing Physiological Properties of Oleamide

Following its identification, synthetic oleamide was tested on rats. The synthetic compound was injected intraperitoneally into rats in doses of 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. Two hours after the lights cycled off (in a 12-hour light/12-hour dark cycle) the low doses of 1 and 2 mg produced no overt effect on behavior. Doses of 5 mg or more, however, induced long-lasting motor quiescence, with closed eyes and sedate behavior characteristic of normal sleep. Also as in normal sleep, the rats still responded to auditory stimuli with an orienting reflex and sustained attention toward the source of stimulation. Behavioral sedation began nearly 4 minutes after injection and lasted 1 hour (for 5 mg), 2 hours (10 mg), or 2.5 hours (for both 20 and 50 mg).

Other known effector molecules were found to work in quantities of approximately 2.8 micrograms. When 2.8 micrograms (10 nmol) of oleamide was introduced into two rats intraventricularly, it induced electrophysiologically monitored sleep,

indicating that the agent acts directly in the brain at a dose comparable with other known effector molecules.

A vehicle of 5% ethanol in saline solution was used to introduce the synthetic carboxamide into the rats. To ensure that neither the vehicle nor oleic acid produced the observed effect, each was introduced in the rats and no overt behavioral effect or modification of the spontaneous sleep–wake cycle occurred. *Trans*-9,10-octadecenoamide exhibited similar, though reduced, effects compared to those of the *cis* isomer. At 10 mg per rat, the effect was 1 hour of sleep instead of the 2 hours noted for the *cis* isomer. Moving the olefin to the 8,9 or 11,12 positions or extending the alkyl chain length decreased its effect markedly. Although the animals still experienced loss of alertness, their eyes remained open and their alertness was only slightly affected.

Recent reports[11] on oleamide hydrolysis in rat brain membrane fraction observed rapid conversion of oleoamide to oleic acid by rat brain membrane fractions. While amide hydrolysis activity was noted in the rat brain soluble fractions, no appreciable ability to hydrolyze the amide to oleic acid was observed in rat pancreatic microsomes and proteinase K. It is conceivable, given this enzymatic hydrolysis of the compound in the brain and the neutral loss of 17 mass units in the initial spectra, that the liberated ammonia also plays a role in the effector function.

Other fatty amide molecules have been reported recently,[12-14] denoting the existence of a class of molecules in which simple variations of a core chemical structure have distinct physiological consequences. Searching for these molecules and uncovering their physiological role will be the basis for further study as this group continues to probe the CSF using continuously evolving techniques in mass spectrometry.

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