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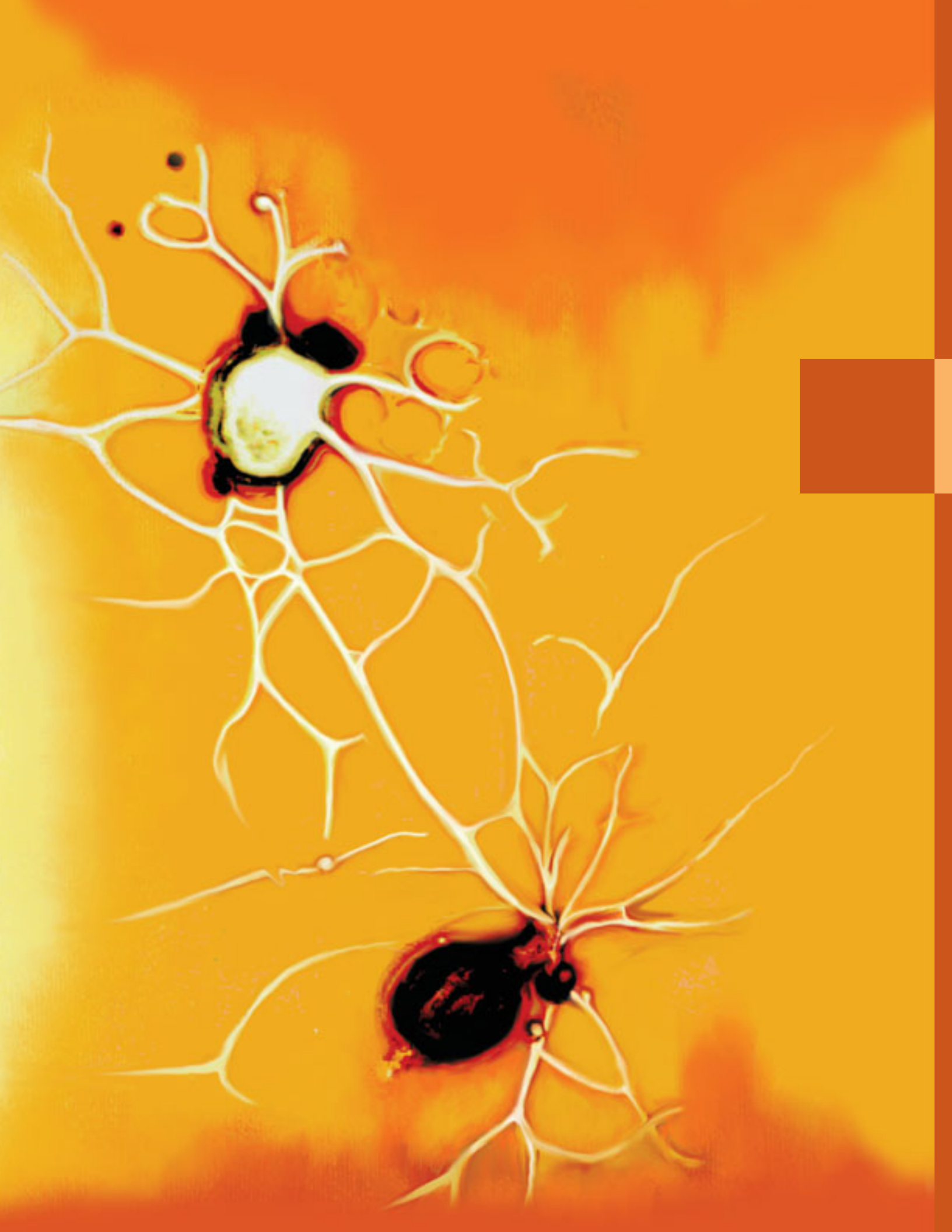

The Chemistry of Thought: NEUROTRANSMITTERS in the Brain

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Spatial and temporal data are critical for knowing how neurotransmitters act upon neurons and, ultimately, how they contribute to larger phenomena such as learning.

Inside a neuron, clusters of molecules shuttle toward a cell membrane tightly packaged within vesicles. The vesicles fuse with the cell membrane, expelling the molecules into the extracellular space. As the molecules reach their targets, electrical currents fly, and the brain hums along.

Without these chemical and electrical transformations, we would not remember the smell of coffee, recognize our child's face, or appreciate a delicate melody. The intricate signals and cascades induce thought, behavior, and memory. Analytical chemists are in on the act of understanding the compounds that drive human contemplation, including the reading of this sentence; what will be remembered after reading it; and even what emotions it will evoke. The goal of this article is to provide a general overview of the methods used to characterize neurotransmission in the brain, update recent specialized articles on brain chemistry, and complement articles about imaging MS and new electroanalytical methods that examine the role of dopamine in addiction (*1*–*3*).

The brain is composed of nervous system cells, or neurons, which communicate with one another through ports called synapses. Cell–cell communication, or neurotransmission, is embodied through electrical impulses or chemical messengers. Although electrophysiological signaling is an important facet of brain function, we will focus on chemical signaling in the brain and the analytical methods used to study it.

Because analytical instrumentation can be tailored to meet the specific needs of studying brain chemistry, it is important to recognize the categories of information critical for comprehending the intricacies of the mind, namely, chemical, temporal, and spatial information. Better analytical methods have helped researchers characterize the chemicals involved in neurotransmission, but this is only part of the story. Spatial and temporal information are just as critical. Where are neurotransmitters released, and at what rate? Measuring these properties facilitates knowing how neurotransmitters impact target neurons and ultimately how they contribute to larger phenomena such as learning.

Getting in your head

Sampling the brain is quite challenging and is often the critical step, because without adequate sampling strategies, optimization of the analytical technique becomes secondary. In the late 1960s, Roger Guillemin characterized brain chemicals known as "hypothetical hypothalamic hypophysiotropic factors". In his 1977 Nobel lecture, he recounted the initial challenges as his group began its search.

Simple reasoning and early chemical confirmation led to the hypothesis that these unknown substances would be small peptides. After several years of pilot studies . . . it became clear that characterizing these hypothalamic hypophysiotropic substances would be a challenge of (originally) unsuspected proportions. Entirely novel bioassays would have to be devised for routine testing of a large number of fractions generated by the chemical purification schemes; more sobering still was the realization in the early 1960s that enormous amounts of hypothalamic fragments (from slaughter house animals) would have to be obtained to have available a sufficient quantity of starting material to attempt a meaningful program of chemical isolation (4).

"A sufficient quantity" in the 1960s translated into >5 million sheep brains, representing >50 tons of hypothalamic fragments (*4*). In one set of experiments, 300,000 sheep hypothalami were used to isolate 1.0 mg of one of these compounds. The practice of late has been to use a single neuron to identify neurotransmitters and neuromodulators—this is 15 orders of magnitude smaller than the sample described above. However, even today, a large population of cells is frequently harvested, a homogeneous mixture produced (as performed by Guillemin in his qualitative search for chemical factors), and the mixture analyzed for the molecules of interest. Although this method is effective for characterizing compounds found in large populations of cells, it becomes problematic when only a few cells in an otherwise homogeneous sample demonstrate drastic differences (often the case in medical disorders), which can lead to sampling bias when the results are extrapolated to single cell values (*5*).

On the flip side, one limitation of single-cell analysis is throughput. Technology issues aside, if a method existed that could analyze the complete cellular complement of a single cell in only 1 s, it would still take >100,000 years of continuous study to analyze the approximate number of cells in the human brain! Hence, multiplexing single-cell methods should prove beneficial—the human genome project was completed only after the creation of such multiplex technology. Yet, other problems, such as data storage and automated methods for handling fragile tissues, would remain.

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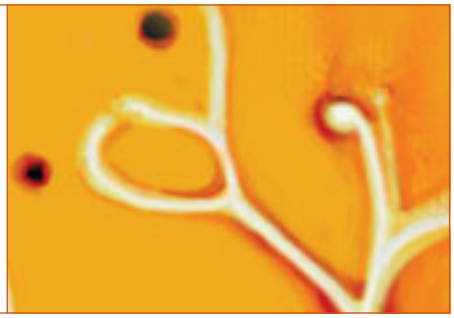

The main sampling strategies for the brain are in vivo and in vitro methods, each of which possesses specific advantages. In vitro methods are usually simpler, though they often require a recently deceased organism and an experienced dissector. Although most in vitro methods acquire a snapshot of the chemical environment of the tissue of interest, in vivo techniques complement the largely chemical and/or spatial information obtained in vitro by providing a sampling method that is ideal for time-dependent studies.

An example of an in vivo sampling method is microdialysis, in which a 1-mm-long, 100-μm-diam probe with a fixed, semipermeable dialysis membrane is inserted into a living organism while a constant, perfused flow of fluid travels into and out of the probe (*6*). Molecules outside of the neurons travel toward the probe because of the concentration gradient. As the flow exits the probe, a variety of analytical methods can be used to analyze the brain chemicals. This sampling method also provides spatial information, generally on a scale >200 μm, so that

different areas of the brain can be probed. Infusing pharmacological substances in the dialysis medium also allows their effects on specific tissues to be determined. Microdialysis has been an effective workhorse in brain sampling in recent decades.

Regrettably, in vivo dialysis possesses limitations due to the nature of dialysis itself, because the chemical concentration gradient drives collection and the invasive nature of the procedure may cause significant tissue damage and potentially misleading readings. Although in vitro sample recoveries are generally <10%, they can be higher with low-flow approaches. An older method known as push–pull perfusion sidesteps dialysis and directly collects sample from the extracellular space. Recently, push–pull perfusion has been miniaturized and was used to collect glutamate from the striatum of an anesthetized rat; flow rates were as low as 10 nL/min, and recoveries were up to 80% (*7*). These attributes can make the difference when detecting low concentrations of neurochemicals, and the diminutive physical size and flow rate minimize tissue damage. However, most current analytical methods require larger sample volumes, hampering the impressive temporal resolution possible from push–pull perfusion. We expect that emerging analytical techniques will use ever-smaller sample volumes, making push–pull perfusion promising.

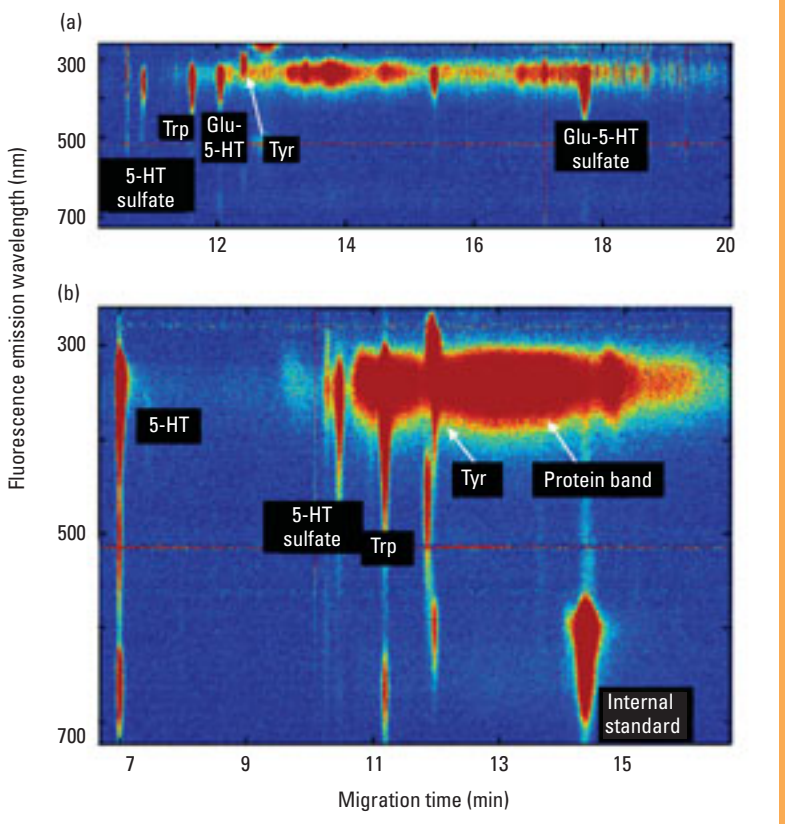

FIGURE 1. (a) 5-HT incubation of *Aplysia californica* cultured neurons results in formation of the novel catabolites 5-HT-sulfate, γ-Glu-5-HT, and γ-Glu-5-HT-sulfate. The blue areas indicate low signal intensity, and red areas indicate high signal intensity. (b) Single-neuron analysis of an *Aplysia* metacerebral cell reveals native presence of O-sulfated 5-HT.

Indoleamines and catecholamines

Of interest are molecules that serve as the chemical messengers of emotion, thought, and memory storage. Unfortunately, there is not one molecule for each of these human categorizations; rather, a range of transmitters differentially affect neuronal response through temporally and spatially distinct mechanisms. Classical chemical messengers include the indoleamines and catecholamines, but molecules such as nitric oxide and large peptide variants have demonstrated important roles in neurotransmission. Although there are many more neurotransmitters, we will focus on these species, which are determined by a suite of techniques representative of those used for other neuroactive compounds.

Because of the chemical complexity of the brain, separations are often required before detection. For measurements of classical neurotransmitters in vitro, CE or HPLC with a sensitive detector for trace analysis, usually fluorescence or electrochemical detection, is often used (*8*). These techniques can be readily optimized and tailored to the specific chemistry under investigation to provide excellent specificity, sensitivity, and a large amount of chemical information. For example, an indole- and catechol-specific, single-cell CE system with wavelength-resolved native fluorescence detection has been used to characterize previously unknown metabolic processes in the brain (Figure 1; *9*, *10*). Although attomole detection limits are possible for some transmitters, such as indoleamines, this detection mode is limited to natively fluorescent (aromatic) transmitters.

Microdialysis is often used for in vivo sampling of classical transmitters and can be coupled off-line to a separation method

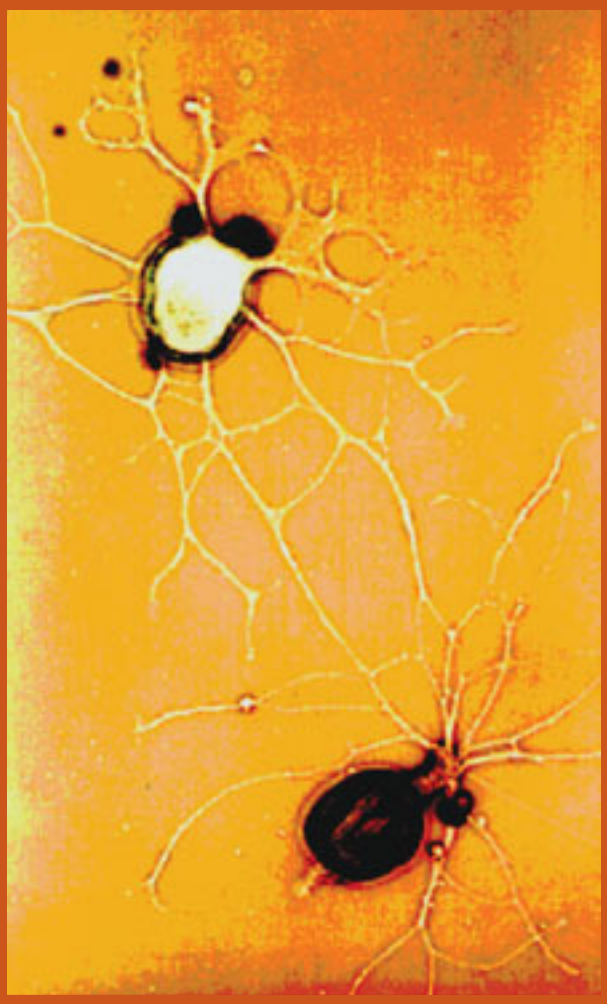

FIGURE 2. Individual *Lymnaea stagnalis* neurons stained with NADPH-diaphorase. The darkened area in the cell at the bottom indicates an NO-positive result. (Adapted with permission from Ref. 24.)

with a sensitive detector for trace analysis. In a rare example using a living human brain, microdialysis in combination with HPLC demonstrated that dopamine release correlates with learning and memory (*11*). However, when microdialysis is coupled off-line to a separation, the volume of the fraction collected (and hence the flow rate through the dialysis probe, often in the low microliter-per-minute regime) usually limits the time resolution of the method, often on the order of minutes (*12*). Capillary separations generally require less sample volume for injection and allow improved time resolution. On-line methods are much preferred when greater temporal information is desired. A time resolution of 10 s was obtained when microdialysis was coupled to CE for analysis of several amino acid neurotransmitters (*13*). This technique demonstrates measurable changes in brain chemistry on the scale of several seconds and is advantageous for tracking neurotransmitter and catabolite levels in the brain's extracellular spaces.

However, for events on a much shorter time scale, such as exocytosis and real-time in vivo neurotransmission of indoleamines and catecholamines, electroanalytical probes are the method of choice (*2*). Since the advent of the carbon fiber microelectrode (*14*), rapid progress has ensued. Once these probes were used to detect electrically induced extracellular dopamine release in a mammalian brain, an entirely new field of electrophysiology opened for exploration. These probes provide excellent temporal information, as the fastest electroanalytical methods to date follow submillisecond events. These probes possess nanomolar sensitivity that rivals the best fluorescence detection. Furthermore, because these probes are frequently orders of magnitude smaller than common microdialysis probes, greater spatial resolution can often be achieved with less tissue damage. Unfortunately, because many compounds in the brain are electroactive, selectivity can be a major hurdle.

In contrast to ultrafast techniques such as amperometry, fast-scan cyclic voltammetry (FSCV) sacrifices orders of magnitude of temporal resolution in exchange for improved selectivity (*15*). With this technique, greater chemical information can be obtained from a complex sampling environment. The distinctive oxidation and reduction potentials of the neurotransmitter of interest are exploited by taking measurements approximately every 10 ms. Common interfering species often possess different oxidation and reduction potentials relative to the indoleamine or catecholamine, and therefore the current originating from the interferences can be better differentiated (*16*). With this novel method, researchers can now follow animal behavior (altered by alcohol or illicit drugs) and correlate certain behaviors with native neurotransmission in various brain regions of freely moving animals. As one particularly exciting example, changes in dopamine dynamics have been measured in drug-seeking behaviors using real-time FSCV (*17*).

Although impressive, FSCV can be affected by common cations that adsorb to the carbon surface and cannot resolve the electrically similar neurotransmitters norepinephrine and dopamine (*18*, *19*). Another method to enhance selectivity is to coat the carbon fiber microelectrode with cation-exchange polymers to reduce interferences from negatively charged molecules; however, temporal resolution decreases because more time is required for analyte diffusion through the layer. Regardless, for rapid in vivo events, electroanalytical probes are the method of choice for acquiring temporal information on known electroactive neurotransmitters in the brains of freely moving animals.

Nitric oxide

Whereas indoleamines and catecholamines have been known to play important roles in neurotransmission for several decades,

NO has been observed only recently in the brain (*20*). Many approaches have been used to attempt detection of this fleeting free radical, including electron paramagnetic resonance (EPR), microchip electrochemical sensors, and fluorescent imaging, with varying degrees of success. Traditionally, NO detection has used either the Griess reaction (*21*) or the oxyhemoglobin reaction (*22*) to form species that cause changes in optical absorption spectra. Not only is this method of measuring NO indirect, but there are issues with selectivity. EPR relies on the spin orientation of unpaired electrons in a magnetic field and can detect NO with spin traps at micromolar detection limits. EPR has detected NO in the rat brain (*23*). Because of the constraints imposed by EPR instrument design, however, little spatial and temporal information can be acquired and fairly specialized equipment is needed.

Histochemical methods have revealed that fixative-resistant NADPH-diaphorase staining correlates to the presence of the NO synthase enzyme (Figure 2; *24*). This works because NADPH is a cofactor used by NO synthase, and the enzyme is particularly resistant to chemical fixation. Although other enzymes use the same cofactor, under optimum (and tissue-dependent) fixation protocols, only neurons with active NO synthase are stained. In situ and immunohistochemical approaches that target NO synthase provide excellent selectivity and spatial characteristics, but they cannot determine whether the enzyme is actively producing NO.

Imaging methodologies have recently emerged as a means to ascertain NO with high sensitivity. A nonfluorescent molecule is loaded into the tissue of interest. The molecule reacts with NO to form a highly fluorescent molecule that can be viewed with fluorescence microscopy. Imaging using noncytotoxic protocols allows for the acquisition of enhanced spatial information with excellent sensitivity—in the best cases, with nanomolar detection limits and submicrometer spatial resolution. Among the most commonly used probes are the series of fluorescent NO indicators, the diaminofluoresceins (*25*, *26*). Unfortunately, improvements are needed to enhance the selectivity of these reagents because cross-reaction with other cellular constituents complicates the signals. These interferences include divalent cations and ascorbic acid compounds, which are often naturally present in concentrations that are orders of magnitude greater than NO itself in the same brain tissues (*27*, *28*). However, for rapid spatial analysis of large tissues, such methods remain an excellent choice.

Another approach is electrochemical sensors that measure NO by either catalytic or direct electrooxidation of NO. In catalytic electrooxidation, a porphyrin film plated onto an electrode interacts with the NO. As with indoles and catechols, selectivity must be carefully evaluated in the specific tissues examined. In direct measurements, NO interacts at the microelectrode surface. Although coating the electrodes with polymeric ion exchangers removes anionic interferences such as nitrite, direct electrochemical sensing may still have specificity issues despite subnanomolar detection limits (*29*). For example, in neuronal populations involved with both serotonergic and nitrergic signaling, electroactive serotonin may exist at a concentration >1000-fold higher than that of NO, which means that high electrode selectivity is needed to detect the NO. Performance issues currently being addressed include sensitivity variations of the microelectrodes as a function of temperature and electrode-to-electrode variability caused by membrane thickness and uneven polymer coating. Furthermore, sensitivities are often measured at concentrations substantially greater than those of physiologically significant ranges (*30*). We envision the design of improved coatings to enhance selectivity for NO without large sacrifices in response time and look forward to selective electrodes that will soon allow sensitive, reproducible real-time monitoring of NO signaling in spatially defined locations in the brain.

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Neuropeptides

If NO is one of the simplest neurotransmitters, the neuropeptides are the largest and most complex category of signaling molecules. With 20 amino acid building blocks and an infinite assortment of linkages, neuropeptides are structurally the largest and most diverse group of neuromodulators. Neuropeptides can range in size from longer hormone sequences like insulin to smaller peptide chains like the enkephalins, a five-amino-acid molecule that inhibits pain pathways in the central nervous system. Often more potent than other neurotransmit-

ters, neuropeptides are subsequently synthesized in smaller quantities and are active at lower concentrations. Neuropeptides are released and are active at micromolar to picomolar concentrations, which are up to a million-fold lower than those of the amino acid neurotransmitters (*12*). These lower concentrations and the chemical diversity of the transmitters, however, make detection a challenge. To measure neuropeptides in specific regions, analytical techniques must be sensitive to attomole masses in microliter sample volumes (*12*).

For the well-characterized transmitters described earlier, method development is intended to improve the spatial and temporal information acquired from existing techniques. Over the past decade, more than 700 new peptides and neuropeptides have been reported in animals ranging from mollusks to humans. For these neuropeptides, methods are required to characterize and localize the spatial and temporal information of these molecules.

Genetic information is changing neuropeptide discovery—genomic databases can now be scanned for potential neuropeptides. Multiple neuropeptides, linked together with enzymatic cleavage sites, are often encoded by a single protein precursor. Neuropeptide precursor sequences can be inferred from genetic strategies, but exact predictions of peptides from genetic information are difficult with current bioinformatic tools (*31*). The processing of specific neuropeptides from a common precursor can differ between cells and at varying points in a cell's development. One additional difficulty is anticipating the posttranslational modifications that will alter the structure and often the activity of a neuropeptide. Because of this laundry list of challenges, MS has become the method of choice to study neuropeptides.

The increased capabilities of MS and small-scale separations have revolutionized neuropeptide discovery. Much of this recent progress has been accomplished with MALDI and electrospray ionization (ESI) MS, which is often coupled to a separation scheme to study homogenized neuronal tissue. In a recent example, central nervous system tissue from *Drosophila melanogaster* larvae was extracted, separated, and analyzed by capillary LC/tandem nano-ESI MS (*32*). Online analysis allowed individual ions of interest to be selected and fragmented as they eluted from the column, even if they coeluted with other peptides. The ability to fragment and sequence these ions enabled the discovery of multiple novel neuropeptides. Of course, homogenization of a complex brain sample means a trade-off in terms of information. The chemical identities of these peptides were successfully determined, but all information regarding the spatial origin of these peptides was lost with homogenization.

A sufficient quantity of material for an analysis can be obtained from a sample as small as an individual cell. One approach that has been successful at discovering hundreds of new neuropeptides is single-neuron analysis by MALDI MS (*33–36*). Analysis of single neurons provides a profile of all of the peptides present at significant concentrations, generating a unique pattern of masses that can be used to recognize a cell. Peptides can be tentatively identified by mass and then further characterized by sequencing (*35*, *37*). These signals can also be correlated with a precursor sequence to determine and confirm prohormone processing.

In addition, individual peptides can be selected, fragmented, and sequenced. For example, a single neuron from the snail *Lymnaea stagnalis* provided a sufficient quantity of material to identify all the peptides' products from both mRNA splice variants of the FMRFamide gene by MS (*38*). MALDI's compati-

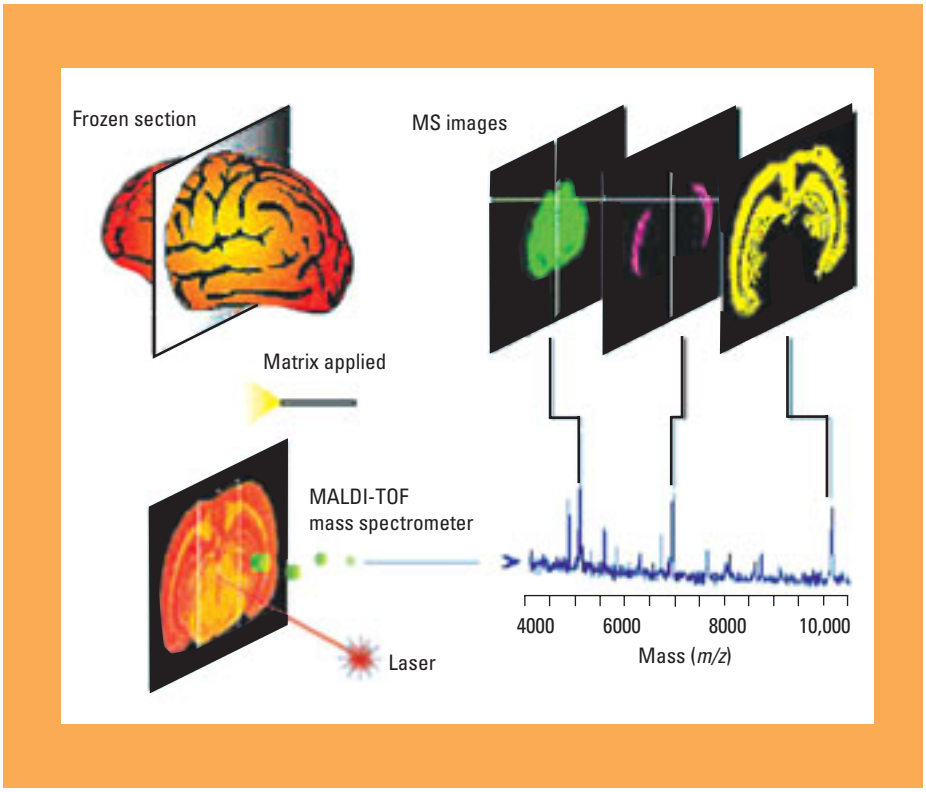


FIGURE 3. The methodology for MALDI imaging MS.

Matrix solution is applied to a tissue slice. The sample is then probed by MALDI-TOF MS; the laser uniformly rasters the sample. The resulting spectra are compiled into intensity maps for specific peptide masses. (Adapted with permission from Ref. 42.)

bility with heterogeneous solid samples allows the analysis of clusters of neurons; imaging of brain slices; and peptide profiling of smaller samples such as individual neurons, connectives, and even vesicles (*33*, *39*, *40*).

How is spatial information on a particular peptide obtained? Two common methods are used for mapping the spatial distribution of a previously characterized neuropeptide: immunohistochemistry, which identifies the location of the peptides themselves, and in situ hybridization, which enables localization of neuropeptide gene transcription by indicating the presence of the precursor mRNA. For in situ hybridization, labeled antisense DNA or RNA probe is hybridized to mRNA within a cellular sample. The probe is detected by emulsion autoradiography (radiolabeled probes), electron microscopy (gold-labeled probes), or light and fluorescence microscopy (antigen-containing probes) (*41*). The main advantages of in situ hybridization are high specificity and precise spatial location of gene transcription. Precursor molecules can be localized to single cells and even to subcellular regions. However, neuropeptides are often transported from one brain region to another, and because the RNA is not necessarily transported, additional information is often needed.

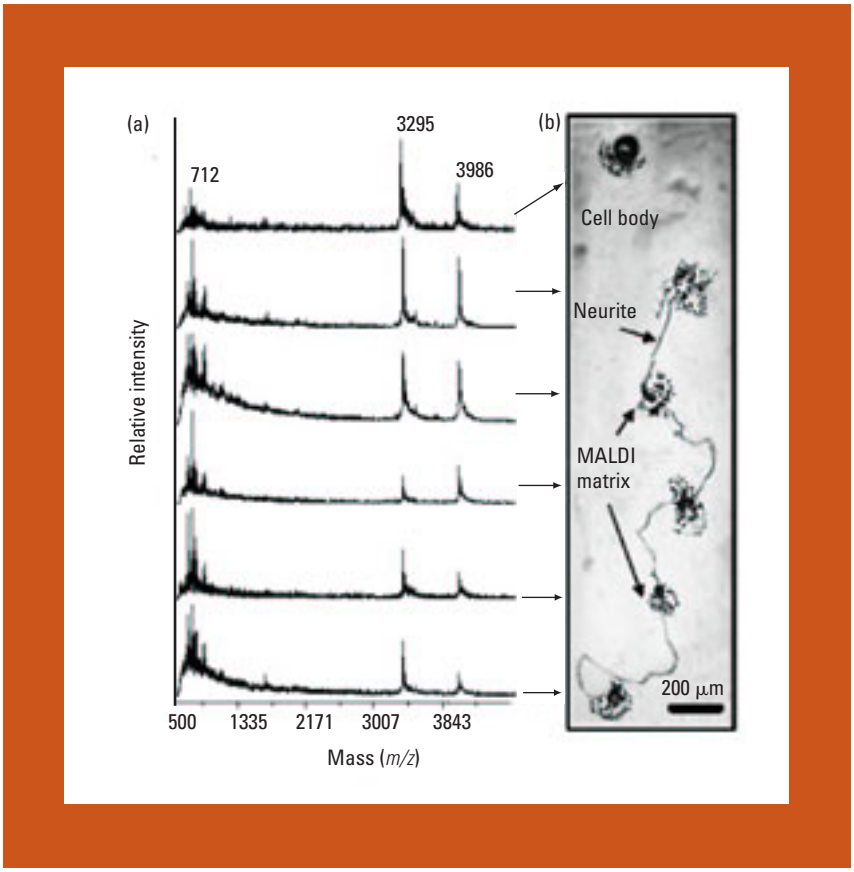


FIGURE 4. (a) MALDI-TOF mass spectra obtained from different cell regions indicated in (b) a transmission light micrograph of a cultured cerebral ganglion neuron stabilized with glycerol. (Adapted from Ref. 44.)

Whereas in situ hybridization examines the spatial distribution of mRNA, immunohistochemistry, also called immunocytochemistry or immunostaining, maps the actual peptide distribution. Antibodies generated against a specific peptide have a high affinity for that peptide and are used to map its expression. These antibodies are labeled with markers and exposed to a slice of tissue or a cluster of neurons, leaving a well-charted map of neuropeptide distribution. Most of the markers used for immunohistochemistry are fluorescently labeled, although radioactive secondary antibodies can also be used (the technique is then named radioimmunolabeling). With either labeling strategy, the technique is sensitive and specific.

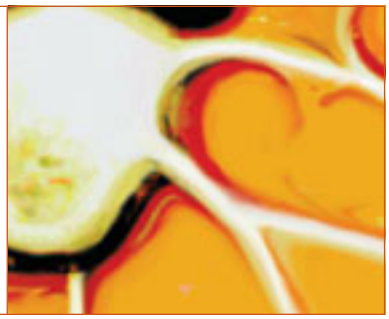
To obtain both chemical and spatial information on neuropeptides in a sample, imaging MS uses MALDI MS to create a 2-D spatial map of peptides in a thin tissue section. As described by Caprioli and co-workers, the brain slice is sectioned and positioned on a MALDI target, and matrix solution is applied (Figure 3; *3*, *39*, *42*, *43*). The laser is rastered across the sample, generating mass profiles at each spot and creating a spatial distribution of masses. Samples as small as a few cells can be profiled (Figure 4; *44*), and relative peptide concentrations can be obtained. Compared to imaging methods described earlier, the spatial information is not as high as that of optical microscopic methods that have submicrometer resolution.

MALDI MS is a valuable tool for the spatial analysis of solid samples, and ESI MS can provide temporal information on liquid samples. In vivo microdialysis has been used to quantify and compare basal versus stimulated extracellular neurotensin concentrations from discrete regions of the rat brain (*45*). The peptides were separated by capillary LC and analyzed by micro-ES tandem MS, which enabled sequencing confirmation for neurotensin. By using capillaries located in particular regions of a brain slice, it may be possible to use ESI MS to produce temporal information on a peptide released from a particular location from the slice.

How will we think about thought in the future?

We have witnessed extraordinary advances in our understanding of the brain over the past decade, but this is only the beginning. Substances not originally believed to play a neurochemical role are being suggested as potential neuromodulators. For example, CO and H_2O_2 may serve as neurotransmitters, and the role of chirality in amino acid neurotransmitters is under investigation (*46*, *47*). Many other classes of cell–cell signaling are ripe

The trend toward smaller, faster, and more information-rich analytical methods will evolve, and new instruments will continue to refine our understanding of thought.



for investigation, including trophins and cytokines. However, intercellular signaling is only a small portion of the brain chemistry needing exploration. Regardless of the molecules themselves, the trend toward smaller, faster, and more information-rich analytical methods will evolve, and new instruments will continue to refine our understanding of thought. The need for advanced analytical methods knows no bounds, and these instruments of the future are only limited by our own brain power. For scientists who scrutinize the world, what could be more natural than to investigate the very process that permits our own exploration?

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