ACS Chemical Neuroscience

Review

pubs.acs.org/chemneuro

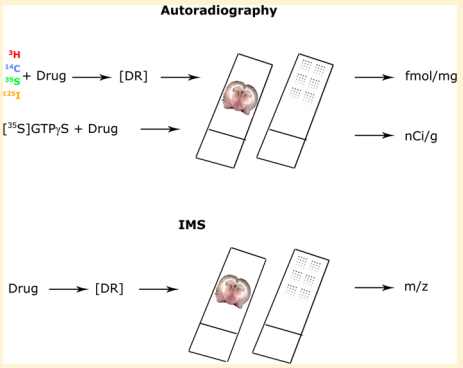
Neurotransmitter Receptor Localization: From Autoradiography to Imaging Mass Spectrometry

Iván Manuel,[†,∥] Gabriel Barreda-Gómez,[§,∥] Estibaliz González de San Román,[†] Antonio Veloso,[‡] José A. Fernández,[‡] M. Teresa Giralt,[†] and Rafael Rodríguez-Puertas*[,†]

[†]Department of Pharmacology, Faculty of Medicine and Odontology, [‡]Department of Physical Chemistry, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Leioa 48940, Spain

[§]IMG Pharma Biotech S.L. Parque Tecnológico de Zamudio, Astondo Bidea, ed. Kabi 612, Módulo 5, 48160 Derio, Spain

ABSTRACT: Autoradiography is used to determine the anatomical distribution of biological molecules in human tissue and experimental animal models. This method is based on the analysis of the specific binding of radiolabeled compounds to locate neurotransmitter receptors or transporters in fresh frozen tissue slices. The anatomical resolution obtained by quantification of the radioligands has allowed the density of receptor proteins to be mapped over the last 40 years. The data yielded by autoradiography identify the receptors at their specific microscopic localization in the tissues and also in their native microenvironment, the intact cell membrane. Furthermore, in functional autoradiography, the effects of small molecules on the activity of G protein-coupled receptors are evaluated. More recently, autoradiography has been combined with membrane microarrays to improve the high-throughput screening of compounds. These technical advances have made autoradiography an essential analytical method for the progress of drug discovery. We include the future prospects and some preliminary results for imaging mass spectrometry (IMS) as a useful new method in pharmacodynamic and pharmacokinetic studies, complementing autoradiographic studies. IMS results could also be presented as density maps of molecules, proteins, and metabolites in tissue sections that can be identified, localized, and quantified, with the advantage of avoiding any labeling of marker molecules. The limitations and future developments of these techniques are discussed here.



KEYWORDS: *Autoradiography, microarrays, [35S]GTPγS, MALDI, imaging mass spectrometry (IMS)*

Autoradiography, as its name indicates, is based on the use of luminography to generate images of slices of organs or tissues exposed to a radiosensitive film. The development of this technique has gone from the first autoradiographic images obtained from experimental animals after their treatment with radioisotopes *ex vivo*[1] to the current images of the whole animal obtained by phosphor imager equipment.[2] Several variants of autoradiography have been developed, but all of them are based on a detection method that provides spatial information about the distribution of radioactive signals after being exposed to photosensitive films or to a photographic emulsion. The sections are usually obtained either from animals treated with a radioligand (*ex vivo*) or from tissue sections incubated with a radiolabeled compound *in vitro*.[3−6]

Over the last few decades, new techniques or variants that provide density maps of anatomical images, but using nonradioactive detection methods, such as some mass spectrometric methods, have been developed.[7,8] These techniques, which avoid the use of radioligands, are still limited by some technical problems, which mainly affect both the type of compounds or biological substances that can be detected and the image resolution. These limitations, together with the standardization achieved by quantification in autoradiography, have made this technique an irreplaceable procedure for the anatomical quantification of proteins such as membrane receptors or neurotransmitter transporters, which is necessary for a multitude of pharmacodynamic studies.[9] Radioactive labeling can also be used to determine the spatial profile of different compounds that recognize molecules, such as DNA and RNA, by *in situ* hybridization (e.g., specific sequences of nucleotides) or even to recognize proteins using immunohistochemistry. However, although these techniques could also be considered autoradiographic methods, the present work is focused on the application of autoradiography in pharmacodynamic studies for drug discovery and the evaluation of imaging mass spectrometry (IMS) as a new complementary method.

In particular, this review concerns the application of autoradiography techniques to the study of neurotransmitter receptors, an important class of drug targets.

The anatomical distribution of the myriad subtypes of both superfamilies of neurotransmitter receptors, ionotropic and G protein-coupled receptor,[10−14] has been characterized to a great extent thanks to the use of autoradiography. In relation to ionotropic receptors, the nicotinic for acetylcholine, ionotropic

Received: November 5, 2014
Revised: January 30, 2015
Published: February 3, 2015

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

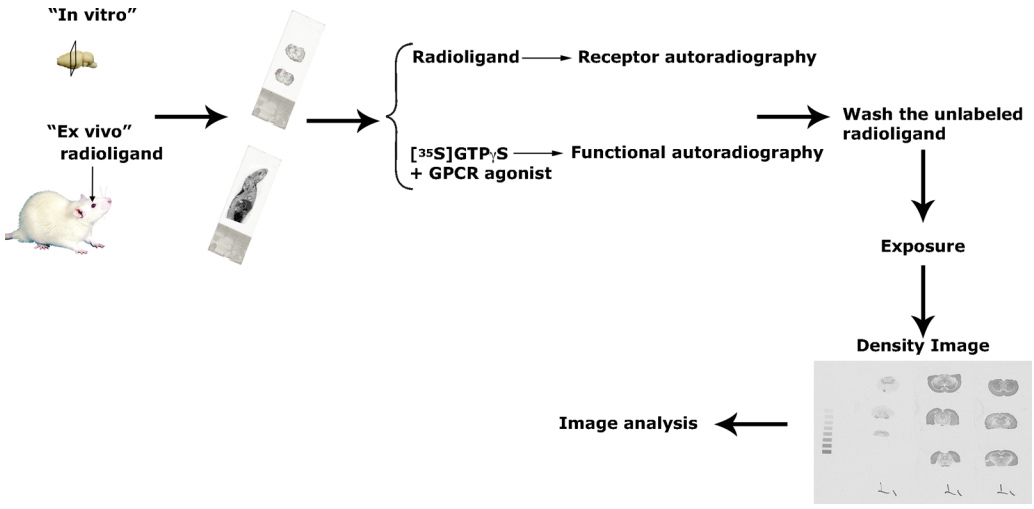


Figure 1. Schematic diagram that summarizes the main steps in the different autoradiography formats: *in vitro*, *ex vivo*, and functional autoradiography. For *ex vivo* autoradiography, the radioligand is administered directly to the live animal. Then, the tissue is dissected, cut, and mounted on slides in a cryostat. The slides are incubated afterward with the radioligand only in the case of *in vitro* autoradiography. Functional autoradiography is a subtype of *in vitro* autoradiography. The following steps are common for all variants of autoradiography and include washing of the unlabeled radioligand and exposure to a radiosensitive plate such as a radioisotope-sensitive film to obtain the density images that are later analyzed by computer software.

glutamate, and purinergic P2X receptors are examples of channels through which diverse cations flow, whereas other receptors, such as $GABA_A$ subtype for gamma-aminobutyric acid, are permeable to anions.[11−13] Some second messengers, such as cyclic nucleotide-gated channels, also activate some ion channels and can be identified by autoradiographic methods. The other family of neurotransmitter receptors is constituted by the G protein-coupled receptors (GPCR).[14,15] The rhodopsin family is the most abundant and is composed of monoamine, peptide, and prostaglandin receptors as well as odorant, visual, and some types of viral receptors. Therefore, the molecules that recognize the different subtypes of GPCR are potential markers, when labeled by radioisotopes, to localize their receptors by autoradiography. These molecules can be endogenous substances such as neurotransmitters as well as synthetic compounds that bind to their specific binding sites by interacting with amino acid residues usually located at the transmembrane domains that constitute the binding pocket. The intracellular loops of this family of receptors are coupled to guanine nucleotide binding proteins (G protein).[16]

Autoradiography can also be used to quantify and localize the incorporation of radioactively labeled GTP analogues in the α subunit of G protein. Thus, functional autoradiography has allowed the localization of the activity or effects elicited by GPCR agonists by quantifying the increase in the labeling with [^{35}S]GTPγS. Figure 1 summarizes the main steps in the different autoradiography formats: *in vitro*, *ex vivo*, and functional autoradiography. For *ex vivo* autoradiography, the radioligand is administered directly to the live animal. After euthanizing the animal, the tissue is dissected, cut, and mounted on slides in a cryostat. However, the slides are incubated with radioligand only for *in vitro* autoradiography. Functional autoradiography could be considered a subtype of *in vitro* autoradiography. The following steps are common for all variants of autoradiography and include washing of the unlabeled radioligand and exposure to a radiosensitive plate such as a film to obtain the density images that are then analyzed by computer software (Figure 1).

Studies of the anatomical distribution of radioligand binding sites and the activation of G proteins by specific neurotransmitter receptors are the main *in vitro* applications of autoradiography. The advantages and limitations of each procedure and the present and possible future developments of the technique are discussed.

LOCALIZATION OF NEUROTRANSMITTER RECEPTORS BY AUTORADIOGRAPHY

The anatomical localization of neurotransmitter receptors was one of the first and most widely used applications of autoradiography. It is common to perform the first pharmacodynamic experiments for the pharmacological characterization of a compound in cell membrane homogenates of a dissected tissue because it is faster and more economical. However, the microscopic information about the density of binding sites is obtained only by the use of autoradiography in tissue sections, and its use is, therefore, also necessary for small-sized regions that cannot be efficiently dissected.

Furthermore, GPCR genes represent up to 1% of the total genome of mammalians. In the human genome, it is estimated that approximately 1000 genes for GPCR are expressed. The high number and diversity of receptors has made the GPCR one of the principal targets for drugs, especially in the central nervous system (CNS).[17] The development of novel high-throughput screening (HTS) techniques is allowing a large number of molecules synthesized by the pharmaceutical industry to be pharmacologically characterized. Autoradiography is also becoming a HTS technique, and its use is moving toward nonradioactive methods.

***Ex Vivo* Autoradiography.** The use of radioligands and the analysis of binding sites in tissue membrane homogenates have allowed the discovery and pharmacological characterization of multiple types of receptors for neurotransmitters and hormones for many years. These experimental approaches presented a significant limitation regarding the anatomic information that can be obtained until the development of the whole body autoradiography technique by Ullberg in 1954,[18] based on

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

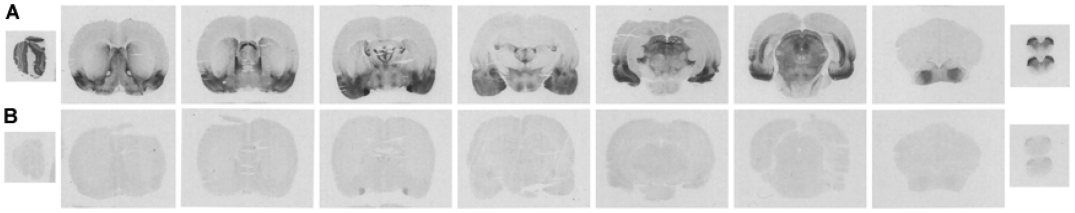


Figure 2. Example of *in vitro* autoradiography images. The ability to identify receptors for neuropeptides is usually limited by the availability of specific compounds that can be labeled with [^{3}H]. It is common to use endogenous neuropeptide labeled with [^{125}I] isotope. The image shows the binding of [^{125}I]galanin (0.1 nM) to coronal sections of Sprague−Dawley rats. Note the specific binding (A) in areas of the olfactory bulb, lateral olfactory tract, mesencephalic nuclei, and cognitive pathways (ventral hippocampus, entorhinal cortex, and medial septum). The nonspecific binding (B) was defined in the presence of a large mass excess of unlabeled galanin (1 μM).

previous studies in animals on the administration of radioisotopes. He treated mice with ^{35}S-penicillin, and tissue sections were exposed to a film.[18] Since then, this technique has been improved with new protocols, equipment, and image analysis software. Basically, the whole body autoradiography technique consists of administering a molecule labeled with a radioactive isotope (such as ^{14}C, ^{3}H, and ^{125}I) to an animal. After euthanizing the animal, the whole body is quickly frozen; optionally, it can be embedded in inert polymers, such as carboxymethylcellulose. Sections around 30−60 μm are obtained in a heavy-duty research grade cryostat for whole body sections (Leica CM 3600XP). These sections can be mounted onto large slides or even on adhesive tape and are then dehydrated and exposed to X-ray films or to phosphor-imaging plates, together with the corresponding standards. The images are calibrated, transforming the optical densities into radioactive units per weight (usually nanocuries per gram of tissue equivalent, nCi/g t.e.), and then the optical density of the different tissues of interest is quantified using image analysis software.[6,19]

The main advantage when comparing whole body *ex vivo* autoradiography to *in vitro* autoradiography is that it enables the distribution of a drug in all organs and tissues to be studied simultaneously. However, both types of autoradiography enable the microscopic localization of drug binding sites in discrete areas, such as the layers of the cerebral cortex, that cannot be clearly dissected to perform binding studies in tissue homogenates. Then, it is possible to obtain very valuable information on pharmacokinetic parameters regarding the absorption, distribution, and excretion of the compounds. However, the pharmacokinetic processes can also modify the calculation of the pharmacodynamic parameters in *ex vivo* autoradiography, such as the maximal density of receptors (B_{max}), which can be better estimated by *in vitro* experiments.

The exposure time of the sections to films depends not only on the activity of the radioligand but also on the density of the binding sites in the analyzed tissue slice. The sophistication of the exposure methods with phosphor-imager systems permits the images to be obtained within a few days, or even hours, in comparison with the weeks or months that are necessary with beta radiation sensitive films. The exposure time is a factor that restricts the use of short-lived isotopes, such as ^{90}Y, ^{11}C, or ^{18}F, but the main limitation is the sectioning of the tissue within the short time window (a few hours) before the counts decay, especially with ^{11}C.[20] Radioisotopes with a longer decay factor, such as ^{125}I, ^{3}H, ^{35}S, or ^{32}P, are a better choice for *in vivo* studies. One of the limitations of *ex vivo* autoradiography is that the technique cannot distinguish directly between specific and nonspecific binding sites. A limitation common to all methods based on the detection of radioligands is that they involve the modification of the native ligand structure, which may change the affinity and therefore the selectivity of the original molecule. Moreover, in *ex vivo* autoradiography, the possible formation of metabolites, which can have different pharmacodynamic properties from those of the original drug, also exists. Additionally, the radioactive contamination of the equipment and organic material and the need for authorized laboratories and workers to carry out the experiments are important handicaps for the use of whole body autoradiography. In this context, the section thickness is restricted by the cryomicrotome, and this feature is important to define the use of the right standard scales that are necessary for image calibration and, therefore, for the evaluation of the radioligand concentration in each tissue or organ.

Microautoradiography is a smaller variant of quantitative whole body autoradiography that was developed during the 1960s.[21] Microautoradiography consists, as does *ex vivo* autoradiography, of the systemic administration of some molecules labeled with radioisotopes (usually ^{3}H, ^{14}C, ^{35}S, or ^{125}I). Then, the animal is dissected, as in *ex vivo* autoradiography, and frozen. Next, the frozen tissue is cut into 4 μm sections. These cryosections are mounted on photographic emulsion-coated slides, which are placed in a dark box in the presence of desiccants for a period of time that is empirically determined. After the exposure period, the entire slices are developed following a similar procedure as that for photographic films.[21,22] This technique has been used to identify the cellular targets for drugs or endogenous metabolites and has contributed to the characterization of new drugs, providing valuable microscopic information about the distribution of receptors.[23,24]

These techniques provide a high-resolution tool for pharmacological research to localize ligand binding sites at cellular and subcellular resolution.[25−27]

***In Vitro* Autoradiography.** *In vitro* receptor autoradiography was one of the first molecular techniques used to observe the localization of the receptors or transporters in sections of biological samples and consists of the incubation of tissue slices with radioligand. Autoradiography of tissue sections has similar experimental requirements as those of membrane homogenates in relation to the properties of ligand−receptor interactions (e.g., affinity, selectivity, nonspecific binding) and incubation conditions (temperature, pH, time) as well as in the pharmacological analysis of the results. During the screening and development of drugs, it is necessary to pharmacologically characterize their affinity by calculating parameters such as the dissociation constant (K_d) and the maximal number of binding sites in a tissue (B_{max}). In addition, *in vitro* differs from *ex vivo*

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

autoradiography in that there is the possibility of controlling other factors that may modulate the specific binding of a compound to the target protein or to nonspecific binding sites. Therefore, experimental factors such as variations in pH, temperature, or incubation time can be specifically analyzed to obtain the optimal incubation conditions for each radioligand.

These radioligand binding techniques are useful in several scientific areas, but they became especially important in biomedicine, where they have been employed for the development of new molecules or drugs for therapeutic use. Moreover, autoradiography has allowed the distribution of a large number of receptors to be mapped as well as provided information about some aspects of their physiological role.

As was commented on above, receptor autoradiography is based on the analysis of the binding of a radioligand to a receptor for that molecule. The *in vitro* assays are relatively simple, although one must take into account that the peculiarities of each drug or compound could produce nonspecific binding sites to the slide surface, to the coating (gelatin, etc.), or to lipids presents in the tissue. The nonspecific binding is measured in a consecutive slice (on another slide) by incubating it with the radioligand in the presence of a displacing compound. The compound must show a high affinity and selectivity for the receptor and must be used at a concentration high enough to displace or inhibit radioligand binding. Figure 2 shows the images obtained in rat brain in a representative *in vitro* autoradiography experiment with a neuropeptide labeled with [^{125}I] isotope. The images in the lower row show the nonspecific binding of the radioligand that can be observed in each of the slices at the same intensity (Figure 2). Sometimes, the analyzed receptor protein has special requirements in order to be labeled, e.g., presence of Na^+. In general terms, the assay of autoradiography requires (1) preincubation of the tissue sections mounted on slides in the presence of an adequate buffer for a period of time and at a temperature that facilitates the removal of possible endogenous ligands; (2) incubation of the tissue in the presence of a radioligand under the appropriate conditions of temperature, pH, presence of ions, solubility, etc. to produce binding; (3) washing the excess radioactive ligand and immersion of the tissue in cold deionized water to remove salts; and (4) exposure to either a photographic film, a photographic emulsion, or a phosphor-imager plate, after drying the slides in a stream of cold air. It is common to obtain digitalized images that can be calibrated with radioactive standards and analyzed by different software (ImageJ is one of the most widely used) to quantify the parameters of interest by measuring the optical density. The results are usually expressed as femtomoles per milligram or nanocuries per gram of tissue equivalent.

The most common application of *in vitro* autoradiographic labeling of receptors has been to produce quantitative maps of the anatomical distribution of receptor proteins. Therefore, the information obtained from the assay contributes not only to the anatomical and pharmacological characterization of the receptor but also suggests its possible physiological roles. This technique allows the analysis of the receptor in different physiological states and in different stages of development or aging as well as in different stages of a given disease such as Alzheimer's.[28,29] It is also possible to determine some changes in the density of receptors after administration of drugs.[30] This technique can be performed in post-mortem tissue from animals and humans.[31] It is also possible to determine the pharmacological parameters (K_d, B_{max}, and K_i) of a compound in microscopic areas by carrying out experiments of saturation or kinetic inhibition. Moreover, it is also possible to microscopically identify and characterize different receptor subtypes using subtype-selective drugs in specific brain nuclei or areas.[32]

The *in vitro* assays present some clear advantages when compared to *in vivo* experiments: they are simple and rapid, and the radioactive material used can be handled without generating large quantities of contaminated waste. Regardless of the type of ligand used, it is required only that the slices are incubated in the presence of the radioligand with a relatively low specific activity (few nanocuries) and volume (usually at nanomolar concentrations). In addition, the assays have a high reproducibility and reliability index because they are normalized and calibrated using radioactive standards, as previously mentioned. Probably the most striking advantage is being able to obtain high-resolution images of the spatial distribution of the binding sites that are characteristic, for example, of a receptor subtype. These images can be analyzed both qualitatively and quantitatively, but the anatomic resolution may be limited by different experimental factors: the type and intensity of the emission energy of the isotope, the distance between the radiation-emitting source and the emulsion, or the thickness and sensitivity of the emulsion. In addition, another disadvantage is the sensitivity of this technique to pre- and post-mortem phenomena, which influence ligand binding, (e.g., asphyxia, drug treatments, or the degree of tissue preservation). Other limiting factors are the lack of availability of a highly specific radioligand with comparable chemical properties as those of the cold ligand; additionally, the receptor must be present at a sufficiently high density in the tissue.

FUNCTIONAL AUTORADIOGRAPHY FOR G PROTEIN-COUPLED RECEPTORS

Functional autoradiography is a method that measures the first step in a key intracellular signal transduction cascade coupled to neurotransmitter receptor stimulation. Unlike classic autoradiography, this type also provides information about the functionality of the receptors in post-mortem tissues together with the anatomical localization of the G proteins activated by a GPCR and therefore also indicates the density of the activated receptors. Functional autoradiography allows the calculation of the half maximal effective concentration (EC_{50}) and the efficacy, with the latter usually being expressed as the maximal effect. This method, alternatively known as autoradiography of the binding of [^{35}S]GTPγS stimulated by agonist, always consists of the incubation of the slices with the same radioligand to label the activated G proteins, but it does so in the presence of different cold drugs. The classical methods used for measuring the activation of G proteins analyze GTPase activity.[33−35] However, these assays analyze GTPase activity by measuring GTP hydrolysis, which is an indirect activity that can be altered by other factors independent of G protein activation.[36] The binding of the nonhydrolyzable GTP analogue, guanosine 5′-(γ-[^{35}S]thiotriphosphate) or [^{35}S]-GTPγS, directly reflects the activation of the G protein alpha subunit by the receptor, measuring the exchange of GDP for GTP (or [^{35}S]GTPγS) in the G protein (Figure 3). The [^{35}S]GTPγS binding assay has been used in reconstituted systems of purified proteins,[37] in membrane homogenates,[37−40] in cells transfected to overexpress GPCR,[41,42] in immunoprecipitation assays,[43] and in *in vitro* autoradiography.[44]

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

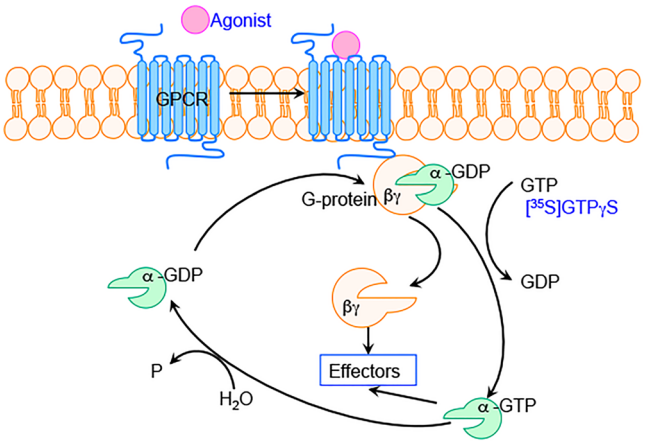


Figure 3. Binding of the nonhydrolyzable GTP analogue, guanosine 5′-(γ-[^{35}S]thiotriphosphate) or [^{35}S]GTPγS, directly reflects the activation of the G protein α subunit by the receptor when it is activated by an agonist compound by measuring the exchange of GDP for GTP (or [^{35}S]GTPγS) in the G protein. The G protein is a trimer that is dissociated, following its activation, to a βγ dimer and the α monomer bound to GTP, both of which are able to activate intracellular effectors such as adenylyl cyclase. The GTP bound to the α subunit is then hydrolyzed to GDP, and the trimeric G protein forms again.

The first evidence of functional receptor−G protein complex in frozen tissue sections was described in the study of Zarbin et al., 1983, in which the authors showed that the binding of some β_2 adrenergic agonists decreased in the presence of GTP analogues.[4] Later, it was demonstrated that the presence of GTP produced a decrease of high-affinity agonist binding sites by converting these sites into low-affinity binding sites.[45] Therefore, they showed that the two states of affinity of the GPCR were preserved, and this provided additional evidence of the interaction between the receptors and G proteins in fresh or frozen preparations. GTP analogues, such as [^{3}H]Gpp(NH)p or [^{35}S]GTPγS, or tritiated GTP itself ([^{3}H]GTP), were used in the first approaches. The first autoradiographic study of localization of guanine nucleotide binding sites was performed in rat brain using [^{3}H]Gpp(NH)p.[46] However, the most frequently used radioligand since then has been [^{35}S]GTPγS. The first study using [^{35}S]GTPγS was to detect G protein activity in the compound eye of *Drosophila*.[47] Subsequently, this technique was adapted by other authors for use with rat brain cryosections.[3,48]

[^{35}S]GTPγS binding assays provide valuable information about the functionality or activity mediated by GPCR. Consequently, the pharmacological parameters that can be calculated are efficiency, expressed as E_{max}, and potency, expressed as EC_{50}. E_{max} represents the highest rate of stimulation produced by a drug in a system or tissue area, whereas the second parameter, EC_{50}, represents the concentration of the drug that induces half of the maximal effect. Determination of the EC_{50} by functional autoradiography requires the incubation of consecutive sections of tissue (instead of tubes used for binding studies in membrane homogenates) at different concentrations of ligand, in a similar way as that in assays to calculate the affinity (K_a or K_d) of a receptor with a radiolabeled drug.

Functional autoradiography allows the activity evoked by a drug in the different discrete areas of a tissue section to be elucidated, and it enables one to classify the compounds as either agonists or antagonists in a specific tissue. The former compounds increase the binding of [^{35}S]GTPγS up to the E_{max} level (total agonists) or below (partial agonists), whereas the antagonists do not produce binding. On the other hand, the inverse agonists inhibit the basal or constitutive binding.[49] Some authors are questioning the cause of the constitutive activity and suggest that if some endogenous ligands are not correctly removed during the experimental procedures then these could contribute to the basal activity. In fact, it has been reported that much of this activity present in rat brain is due to stimulation of adenosine receptors by endogenous adenosine itself.[50]

The type of G protein to which the GPCR is coupled limits the characterization of ligands by functional autoradiography; only the activity of receptors that are coupled to the $G_{i/o}$ family of proteins can be reliably detected. The reason for not detecting other G protein activities (G_s or $G_{q/11}$) is poorly understood. The low densities of the receptors coupled to other subtypes, or of the G_s or $G_{q/11}$ proteins themselves, or low coupling efficiencies might account for this problem.[51] Functional autoradiography can be combined with receptor autoradiography. The anatomical information provided by both methods is often similar for the same GPCR subtype, but sometimes there may be slight differences.[52] This could be because the receptors detected in a specific area may not be active or coupled to $G_{i/o}$ proteins (Figure 4).

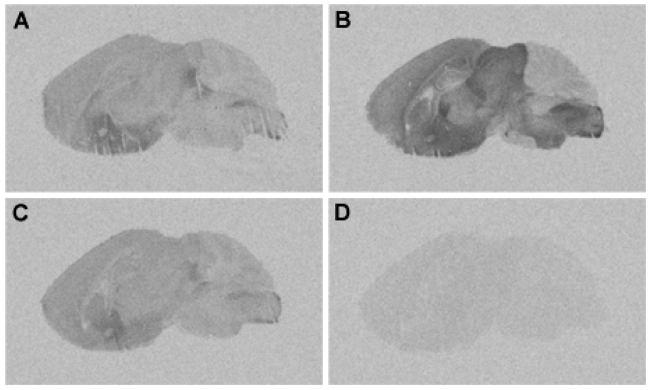


Figure 4. Example of functional autoradiography. [^{35}S]GTPγS binding to mouse sagittal sections in the absence (basal binding; A) or presence of 100 μM carbachol (cholinergic muscarinic agonist) (B) and 100 μM carbachol with 10 μM atropine (cholinergic muscarinic antagonist) (C). As is shown, the effect induced by carbachol is blocked in the presence of the specific muscarinic antagonist, atropine. The nonspecific binding (D) was defined in the presence of 10 μM of unlabeled GTPγS.

The expression or function of GPCR may be altered during development, aging, or by different diseases. For example, alterations to different GPCR densities, such as muscarinic receptors, have been described in Alzheimer's disease.[28] Functional autoradiography is used to evaluate if modifications to receptor densities during disease states or following chronic drug treatments are compensated by a modulation of receptor activity.

Functional autoradiography is complementary to receptor autoradiography with radiolabeled drugs and offers the possibility of quantifying an effect mediated by a GPCR. This functional technique supplies information about GPCRs that are active, whereas conventional autoradiography shows the

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

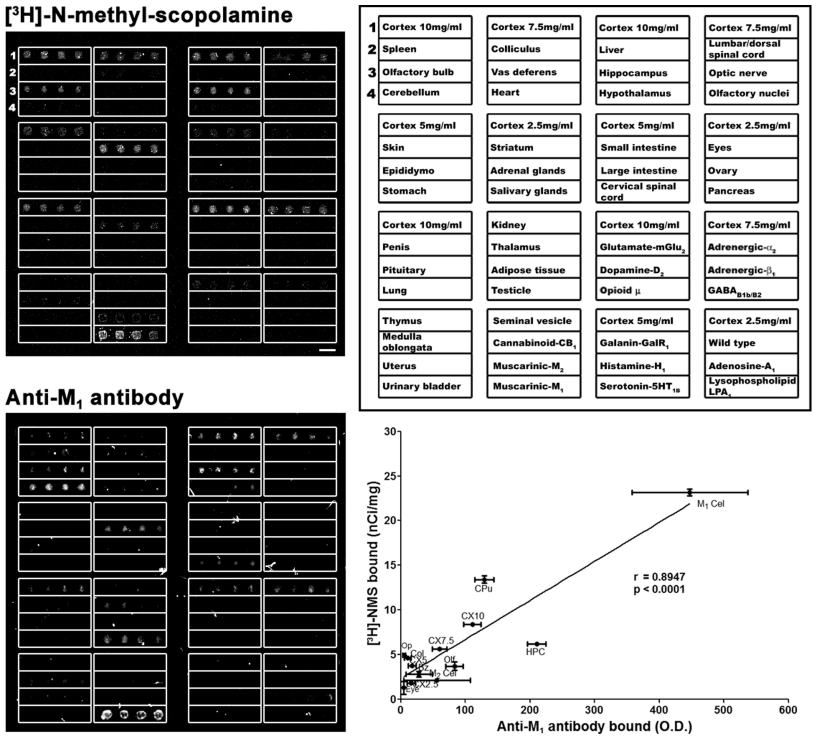


Figure 5. Examples of the information provided by autoradiography of membrane microarrays and the combination with other methods such as immunohistochemistry: [^{3}H]NMS (upper left) and anti-M_1 antibody (lower left) binding to cell membrane microarrays made of homogenates isolated from different tissues and organs of Sprague−Dawley rats and from cells that overexpress a specific GPCR. The cell membranes were printed on slides activated with phosphate groups.[60] In the lower right corner, the density of receptors quantified by autoradiography showed a good correlation with that obtained by immunochemistry (r = 0.8947; p < 0.0001). [^{3}H]NMS is a nonselective antagonist of muscarinic acetylcholine receptors, and the antibody used is designed to specifically recognize the muscarinic M_1 subtype. However, in some tissues, such as cerebellum (line 4 of the first square), the expression measured by the M_1 antibody is higher than the radioligand binding. The combination of different detection methods in consecutive arrays also constitutes a test for different markers. Scale bar = 1 mm.

localization of all receptors, independent of their activation. Therefore, functional autoradiography can help to discriminate the agonistic or antagonistic actions of a drug in each anatomical area, as is shown for the muscarinic receptors in Figure 4. Conventional autoradiography provides information about receptor densities, i.e., the receptor protein that is radioactively labeled by the agonist or antagonist compound. Conversely, functional autoradiography localizes the densities of $G_{i/o}$ proteins coupled to GPCR, allowing one to observe, for example, functional alterations in pathological processes in which receptor density may or may not be modified. The versatility of functional autoradiography, which permits a great variety of different subtypes to be analyzed simultaneously in a single experiment (consecutive slices), is also an advantage in studies directed at comparing differences among GPCR subtypes, as the same [^{35}S]GTPγS radioligand is responsible for the different signal maps.

AUTORADIOGRAPHY ON MEMBRANE MICROARRAYS

Autoradiographic studies in tissue sections require highly qualified staff to process the samples and to analyze the results. This fact makes its automation difficult and therefore limits its use as a high-throughput screening technology. On the other hand, and as has been previously explained, studies in membrane homogenates showed several limitations when used in drug discovery. One of the most important ones is that a relatively large amount of sample is required. Thus, the autoradiography technique can be combined with membrane microarrays to overcome these limitations. In a membrane microarray, each spot is made up of only nanograms of membrane, and the microarray analysis can be automated. In addition, a large number of tissues, cell types, or proteins can be tested simultaneously in a single experiment.

Microarray technology traditionally consists of immobilizing a set of molecules, such as DNA,[53] proteins,[54] carbohydrates,[55] drug-like compounds,[56] cells,[57] tissues,[58] or membrane homogenates,[34] on a solid support, which is usually a microscope slide. Depending on the type of molecule to be captured, the surface of the support can be coated with nitrocellulose, aminopropylsilane, polylysine, avidin, or metals (e.g., gold and silver) or with derivatized with reactive groups such as *n*-hydroxysuccinimide esters, aldehydes, or epoxy groups.

In the case of membrane microarrays, the glass surfaces used to immobilize the cell membranes are typically coated with aminopropylsilane.[34] Other chemical treatments have also been applied such as surfaces coated with cholesterol[59] or phosphate groups.[35,60] Membrane microarrays are made of a collection of different membrane homogenates, isolated from diverse tissues or organs of experimental animal models or even from human samples as well as from transgenic cells that overexpress specific proteins. These microarrays could be employed in drug discovery by the incubation in the presence of candidate compounds being tested for their potential to become therapeutic drugs, which could be labeled fluorescently[34] or radioactively.[36] After incubation with the drug, the unbound compound is washed, and the microarrays are exposed to films,

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

phosphor-imager, or scanners and then analyzed by using specific software. An example is shown in Figure 5, combining radioligand binding with immunodetection of the receptor protein. In general, there is good correlation between the density of radiolabeled receptors (muscarinic) and the immunolabeled receptor protein (M_1 subtype), but it is possible to detect nonspecific binding sites for both the radioligand and the antibody. Therefore, the combination of different detection methods in consecutive arrays also constitutes a test for different markers (Figure 5).

Using GTPγS autoradiography, these microarrays could be used not only to determine the specificity and selectivity of the drug but also to predict the functional response. This kind of functional autoradiography allows one to identify the pharmacological profile of drugs that act through G protein-coupled receptors. In this context, membrane microarrays have been validated for the study of the functional activity of diverse GPCR such as muscarinic, opioid, and cannabinoid receptors with Europium-GTPγS[61] as well as with [^{35}S]GTPγS.[35] Figure 6 shows the application of functional autoradiography in the microarrays of cell membranes to evaluate GTPγS activation induced by a specific agonist in a concentration-dependent manner and simultaneously in different tissues. The example shows the functional autoradiography of CB_1 receptors using a specific cannabinoid agonist, WIN55,212-2. CB_1 receptors are expressed at much lower densities in peripheral tissues, such as the stomach, compared to that in the CNS, and this type of peripheral tissue was included as a reference region or negative control. In fact, one of the most important advantages of the cell membrane microarrays is that this technology permits the simultaneous study of several samples, providing a tremendous amount of information about the primary (desired) and secondary targets (undesired and responsible for adverse effects) of the tested compound in a single miniaturized assay. Therefore, membrane microarray autoradiography is also useful for predicting drug toxicity. This type of microarrays shows additional advantages, such as reducing the quantity of samples, drugs, chemicals, and radioactive residues that are required. On the contrary, the use of membrane homogenates of different brain areas can mask the binding of discrete brain areas or subregions.

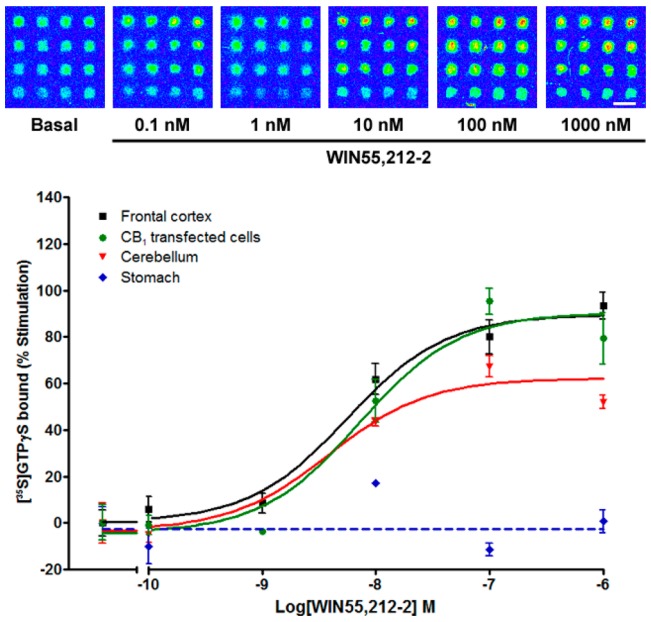


Figure 6. Information provided by the combination of functional autoradiography with membrane microarrays. [^{35}S]GTPγS autoradiographic images obtained from cell membrane microarrays incubated with increasing concentrations of WIN55,212-2 that demonstrate the functional activation of GPCR in these microarrays.[35] CB_1 receptors are expressed at much lower densities in peripheral tissues, such as the stomach, compared to that in the CNS. Therefore, the cell membranes isolated from stomach tissue could be considered here as a negative control. Each array (from top to bottom) consists of four replicates of homogenates isolated from rat frontal cortex (black squares), cells that overexpress the cannabinoid CB_1 receptor (green circles), rat cerebellum (red triangles), and rat stomach (blue diamonds). The cell membranes were printed on slides activated with phosphate groups.[60] The graph shows the concentration−response curves obtained to represent the percentage of stimulation over [^{35}S]GTPγS basal binding induced by increasing concentrations of WIN55,212-2 quantified from the autoradiographic images shown above. Scale bar = 1 mm.

New and promising developments in autoradiography are currently being adapted for use in the high-throughput screening of compounds in membrane microarrays composed of multiple spots from different samples, spotted together on a single slide. This technique combines the versatility of radioligand binding in membrane homogenates with the anatomical and visual information provided by autoradiography, reducing the number of samples and radioisotopes to a minimum. Membrane microarray autoradiography could be used in the drug discovery process as the first screen of the pharmacological properties of newly synthesized compounds. Furthermore, functional autoradiography can also be applied to these arrays to obtain valuable information about the possible pharmacological effects of a drug on multiple types of tissues and cells.

The applicability of autoradiography to localize drug targets within a tissue is frequently limited for different reasons, some of them concerning the restrictions and official authorizations that are required in order to manipulate radioactive materials in a laboratory. Another difficulty with autoradiography that is inherent to the radiolabeling of molecules is that the chemical modifications that are necessary for the introduction of the radioisotope sometimes involve a change in the molecular structure of the native ligand that can affect its pharmacological properties (affinity, selectivity). In addition, the detected labeling shows the distribution of the radioisotopes independently of possible metabolic processes or degradation of the compound. Therefore, it is almost impossible to distinguish between the original molecule binding sites and the contribution to the final signal of the possible metabolites. Furthermore, it can take from several days to several weeks of exposure time to films to obtain autoradiographic images of sufficient sensitivity for distribution studies.

The above-mentioned concerns related to the use of autoradiography have increased the interest in the development of other techniques that avoid the use of radiolabeled compounds.

IMAGING MASS SPECTROMETRY (IMS)

Over the past few years, mass spectrometry (MS) has emerged as a potent analytical chemistry technique with pharmacological applications.[62] Moreover, some variations of the method, such as matrix-assisted laser desorption-ionization (MALDI),[63] secondary ion mass spectrometry (SIMS),[64] or desorption electrospray ionization (DESI),[65] allow the identification of

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

molecules by their molecular weight, thereby avoiding the use of labeled compounds, as required for other drug screening techniques. MS permits the simultaneous detection and identification of the compound or drug of interest and its metabolites. Simultaneously, hundreds of other molecules present in the tissue can be detected. The mass and the fragmentation profile are intrinsic characteristics of each molecule that help to match a peak with a particular compound. During the past decade, MALDI, SIMS, and other techniques have been adapted in order to obtain maps of distribution of molecules in tissue sections (usually fresh frozen). These methods are known as imaging mass spectrometry (IMS) techniques and have been applied to determine the distribution of peptides and metabolites in tissue slices as well as lipids and administered drugs and their metabolites.[6,66−70] IMS emerged from the studies on desorption/ionization of organic substances from surfaces[71] and on the identification of intact microorganisms using MS.[72−74] Those initial studies were essential to the further development of the first images analyzing the distribution of proteins and peptides desorbed directly from a tissue.[75−77] At present, IMS is rapidly entering new biomedical fields, for example, research into novel methods for scanning tumor markers in tissue microarrays.[78]

Briefly, the IMS technique starts by covering a frozen tissue section (10−20 μm), usually placed on a slide, with a suitable chemical matrix. Then, it is introduced into the mass spectrometer under high vacuum. The mass spectrometer is programmed to automatically scan the surface of the tissue, and the spectra obtained at each coordinate of the grid are saved for further analysis. These spectra are then aligned by maximizing the correlation with the overall averaged spectrum, normalized using the total ion current, or any other suitable algorithm, and exported to computer software that creates the images of each peak. A color-coded image can be created by different image analysis software. The results can be similar to autoradiograms with regard to their spatial resolution and density map distribution (Figure 7).

The main advantage of MALDI-IMS resides in its ability to draw the distribution of many substances and metabolites in a single experiment, allowing the identification of a large number of molecules by their mass/fragmentation information in m/z databases and, more importantly, without any previous labeling with fluorescent probes or radioactive isotopes. In addition, the time required to obtain the results from a single section in a MALDI-IMS experiment can be as low as a few hours, whereas autoradiography requires many hours or even days or months, depending on which phosphor-imaging equipment is used, the type and activity of the radioisotope, or if tritium and films are used. However, the main problems currently faced by IMS techniques are related to the limited detection of some molecules, such as drugs. The localization of the binding sites of a drug requires a technique able to detect them at nanomolar or femtomolar concentrations. Therefore, the use of IMS to accurately detect and quantify drugs in tissue sections is still less efficient than that of autoradiography, although it is rapidly approaching its level of accuracy. Nevertheless, the previously mentioned features of this technique suggest that IMS will replace autoradiography over the next years, although further development of the equipment, experimental protocols, and analysis methods is required in order to overcome its current limitations.

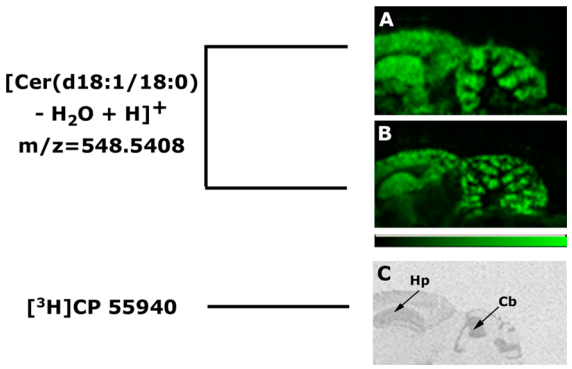


Figure 7. (A, B) MALDI-IMS images representing the distribution of Cer (d18:1/18:0) species collected in sagittal sections of rat brain incubated with (A) WIN55,212-2 (10^{-6} M) and (B) without the drug. The conditions used to acquire the MALDI spectra were as following: 10 shots per pixel, 20 μJ laser energy, spatial resolution of 100 μm, and 100 000 mass resolution. (C) Image of CB_1 receptor autoradiography obtained in the presence of [^{3}H]CP55940, showing the areas where the CB_1 receptor is located in rat CNS. The distribution of the [^{3}H]CP55940 binding sites is similar to the distribution obtained for the m/z 548.5408 peak by IMS.

In a preliminary study, we tried to detect the *in vitro* localization of the binding sites for different drugs such as the cannabinoid compounds WIN55,212-2 or AM251 in rodent brain sections (Figure 7). This was done by incubating the slices in a buffer supplemented with the drug in a range of concentrations that would be able to saturate the target receptors, mimicking the protocol used for autoradiography. We expected that saturating the receptor binding sites would allow us to detect drugs in specific regions of the brain where the receptors are located. The ionization of the drug was previously tested using the same chemical matrix, 2-mercaptobenzothiazole (MBT). In addition, the possible ion suppression of the compound was discarded because we also were able to detect both cannabinoid compounds when deposited together with the MBT matrix directly over the sections. The analysis of the obtained IMS data in these preliminary experiments indicated that, in the case of slices incubated with WIN55,212-2 (10^{-6} M), an intense peak was detected in the mass range of the cannabinoid compound (m/z 548.5508) with a similar distribution as that of the CB_1 receptor. The mass could correspond to the MBT + WIN55,212-2 + H^+, but, after further analysis of a consecutive slice incubated in the same buffer but without WIN55,212-2, we obtained the same peak but with a lower intensity. Therefore, we concluded that it did not correspond to the WIN55,212-2 compound and that it could be a biomolecule present or formed *in vitro* in the tissue (Figure 7). However, another limitation of the IMS technique is assigning the obtained m/z values with specific molecules, which depends on the robustness of the databases. To the best of our knowledge, there are no specific databases for CNS compounds. Nevertheless, we could identify this peak by comparing the results from different databases with a ceramide (d18:0/18:1), which was also described as being upregulated in rodent models of brain injury.[79] That is, the addition of WIN55,212-2 to the buffer would activate CB_1 receptors and induce the production of ceramide. To validate this possibility, it would be interesting to perform MS/MS fragmentation or to try to make the experimental ionization conditions even more restrictive. However, both experimental approaches are complicated with regard to applying them to discrete areas in tissue sections. The

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

IMS technique is evolving rapidly, and the accuracy of molecule databases is continuously improving. Furthermore, physiological information can also help to identify the molecules. Interestingly, cannabinoid activation by the CB_1 receptor could induce sphingomyelin hydrolysis, producing ceramides, at least in glioma and glial cells.[80,81] Further future developments and experiments will provide information on how useful IMS is for obtaining pharmacological information similar to that obtained by the autoradiography of radioligands.

CONCLUSIONS

The incubation of tissue sections with radioligands and the subsequent analysis of the specific binding sites by image analysis systems has been a very useful and unique technique to obtain autoradiograms that indicate the distribution of the targets for drugs, commonly, neurotransmitter receptors and transporters. During the past decade, the technique has been adapted in order to obtain information related to the activity of $G_{i/o}$ protein-coupled receptors, i.e., to quantify the effect, with anatomical resolution, that a drug is able to induce in specific areas of a tissue. This adaptation has allowed the study of the effect of compounds acting through GPCR without labeling them, and only [^{35}S]GTPγS is required. New and promising developments in autoradiography are currently being applied for the high-throughput screening of compounds in membrane microarrays composed of multiple spots from different tissues from experimental animals or humans as well as from cells in culture, all spotted together on a single slide. Autoradiography of cell membrane arrays combines the versatility of radioligand binding in membrane homogenates with the anatomical and visual information provided by autoradiography, reducing the quantities of samples and radioisotopes needed to a minimum. Membrane array autoradiography will accelerate the drug discovery process, principally as a first screen of the pharmacological properties of newly synthesized compounds. Furthermore, functional autoradiography can also be applied to these arrays, providing valuable information about the possible pharmacological effects of a drug on multiple types of tissue and cells.

Additionally, autoradiography is facing some restrictions that are inherent to the use of radioactive isotopes, and some companies that developed reagents and materials for autoradiography have tried to substitute them with fluorescent or luminescent labels, which has been met with relative success because reaching quantification in the femtomolar range of radioisotopes is complicated. Obtaining high resolution is one of the major challenges facing IMS if it is to be used as a substitute for autoradiography in the future. Also, the identification of compounds without any labeling, just by their exact molecular mass and ionization, is necessary for the success of IMS. This is not an easy task, but important research being carried out by multidisciplinary teams of pharmacologists, biochemists, synthetic chemists, and physical chemists is leading to the development of new mass spectrometers based on MALDI and SIMS methods and to new ways of ionizing the molecules so that they can be specifically recognized in the detectors. The future of autoradiography probably lies in IMS, and drug discovery departments should begin to take this into consideration and become familiar with this technique. However, it is necessary to improve the method for the detection of femtomolar concentrations of drugs in both tissue sections and in membrane microarrays. Some of the factors precluding the use of IMS for neurotransmitter receptor localization include avoiding ion suppression of the compound and determining the right assignment of the obtained m/z values to specific molecules, which depend on the accuracy and specificity of large databases. Specific databases for the CNS could also improve the interpretation of IMS results related to molecules involved in neurotransmission. The assignment could also be validated by combining different methods such as MS/MS fragmentation coupled to HPLC and by increasing the selectivity of the experimental ionization conditions, e.g., developing selective matrices for each type or chemical family of molecules. The application of IMS to different experimental models can provide physiological information, which is helpful not only for the identification of the molecules but also for understanding their modulation.

Future developments of the IMS technique could lead to it being able to fulfill most of the screening requirements for the initial pharmacological characterization of new drug candidates. Until that occurs, autoradiographic techniques will continue to be used for their initial purpose, which is that of anatomically localizing drug targets.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rafael.rodriguez@ehu.es.

Author Contributions

[‖]I.M. and G.B.-G. contributed equally to this work.

Funding

This work was supported by grants from the Basque Government, IT584-13, and Spanish Government, Ministry for Health, I.S.C.III PI 10/01202, co-funded by European Research Development Fund.

Notes

The authors declare no competing financial interest.

KEY CONCEPTS

B_{max}: The concept of B_{max} is a measure of the density of receptor molecules in a tissue, and in the techniques of receptor binding, it is equivalent to the bound compound when all of the receptors are occupied by a saturating concentration of the radioactive ligand. Receptor binding studies are used to calculate the affinity (determined by K_d) of a drug for a receptor as well as the maximal density of binding sites (B_{max}). It is usually determined in a saturation experiment, but it can also be calculated for a nonsaturating concentration (L) of the radioligand by the occupancy theory when the dissociation equilibrium constant of a drug for the receptor (K_d) is previously known, using the following equation: bound = $B_{max}[L]/K_d + [L]$.

G protein activation cycle: When GPCR are activated, they induce the exchange of GDP for GTP in the α subunit of the G protein that is dissociated to interact with intracellular or membrane effectors (e.g., enzymes and ion channels). The activation cycle is completed when the GTPase of α subunit hydrolyses GTP into GDP and restores the initial inactive conformation as well as its affinity for $\beta\gamma$ complex. In the presence of the nonhydrolyzable analogue of GTP, guanosine 5′-(γ-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS), the cycle is stopped and thus allows one to quantify the activity of G proteins in basal and activated states. The binding of [^{35}S]GTPγS indicates the activation of the G protein α subunit by the receptor when it is activated by an agonist.

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

E_{max}: E_{max} is the maximal effect or response elicited by a drug. The EC_{50} for *in vitro* experiments (ED_{50} for *in vivo*) is the dose of agonist that produces 50% of the maximal response.

REFERENCES

(1) London, E. S. (1904) O fiziologopatologicheskrom znachenii emanastii radya (Physiopathological importance of radium emanation). *Russ. Vrach 3*, 869−72.

(2) Kanekal, S., Sahai, A., Jones, R. E., and Brown, D. (1995) Storage-phosphor autoradiography: a rapid and highly sensitive method for spatial imaging and quantitation of radioisotopes. *J. Pharmacol. Toxicol. Methods 33*, 171−8.

(3) Sim, L. J., Selley, D. E., and Childers, S. R. (1997) Autoradiographic visualization in brain of receptor-G protein coupling using [^{35}S]GTP gamma S binding. *Methods Mol. Biol. 83*, 117−32.

(4) Zarbin, M. A., Palacios, J. M., Wamsley, J. K., and Kuhar, M. J. (1983) Axonal transport of beta-adrenergic receptors. Antero- and retrogradely transported receptors differ in agonist affinity and nucleotide sensitivity. *Mol. Pharmacol. 24*, 341−8.

(5) Sóvágó, J., Dupuis, D. S., Gulyás, B., and Hall, H. (2001) An overview on functional receptor autoradiography using [^{35}S]-GTPgammaS. *Brain Res. Rev. 38*, 149−64.

(6) Solon, E. G., Schweitzer, A., Stoeckli, M., and Prideaux, B. (2010) Autoradiography, MALDI-MS, and SIMS-MS imaging in pharmaceutical discovery and development. *AAPS. J. 12*, 11−26.

(7) Spengler, B., Hubert, M., and Kaufmann, R. (1994) *MALDI ion imaging and biological ion imaging with a new scanning UV-laser microprobe*, Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL, May 29 −June 3.

(8) Stoeckli, M., Chaurand, P., Hallahan, D. E., and Caprioli, R. M. (2001) Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. *Nat. Med. 7*, 493−6.

(9) Veloso, A., Fernández, R., Astigarraga, E., Barreda-Gómez, G., Manuel, I., Giralt, M. T., Ferrer, I., Ochoa, B., Rodríguez-Puertas, R., and Fernández, J.A. (2011) Distribution of lipids in human brain. *Anal. Bioanal. Chem. 401*, 89−101.

(10) Clapham, D. E., Montell, C., Schultz, G., Julius, D., and International Union of Pharmacology (2003) International Union of Pharmacology. XLIII. Compendium of voltage-gated ion channels: transient receptor potential channels. *Pharmacol. Rev. 55*, 591−6.

(11) Moroni, M., Meyer, J. O., Lahmann, C., and Sivilotti, L. G. (2011) In glycine and GABAA channels, different subunits contribute asymmetrically to channel conductance via residues in the extracellular domain. *J. Biol. Chem. 286*, 13414−22.

(12) North, R. A. (1996) P2X receptors: a third major class of ligand-gated ion channels. *Ciba. Found. Symp. 198*, 91−105.

(13) Cascio, M. (2004) Structure and function of the glycine receptor and related nicotinicoid receptors. *J. Biol. Chem. 279*, 19383−6.

(14) Hermans, E. (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol. Ther. 99*, 25−44.

(15) Rosenbaum, D. M., Rasmussen, S. G., and Kobilka, B. K. (2009) The structure and function of G-protein-coupled receptors. *Nature 459*, 356−63.

(16) García-Jiménez, A., Cowburn, R. F., Winblad, B., and Fastbom, J. (1997) Autoradiographic characterisation of [^{35}S]GTP gamma S binding sites in rat brain. *Neurochem. Res. 22*, 1055−63.

(17) Eglen, R. M., Bosse, R., and Reisine, T. (2007) Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening. *Assay Drug. Dev. Technol. 5*, 425−51.

(18) Ullberg, S. (1954) Studies on the distribution and fate of ^{35}S-labelled benzylpenicillin in the body. *Acta Radiol., Suppl. 118*, 1−110.

(19) Schweitzer, A., Fahr, A., and Niederberger, W. (1975) A simple method for quantitation of ^{14}C-whole-body autoradiograms. *Appl. Radiat. Isot. 33*, 329−33.

(20) Planas, A. M., Crouzel, C., Hinnen, F., Jobert, A., Né, F., DiGiamberardino, L., and Tavitian, B. (1994) Rat brain acetylcholinesterase visualized with [^{11}C]physostigmine. *Neuroimage 3*, 173−180.

(21) Stumpf, W. E. (2012) Drugs in the brain—cellular imaging with receptor microscopic autoradiography. *Prog. Histochem. Cytochem. 47*, 1−26.

(22) Stumpf, W. E. (2005) Drug localization and targeting with receptor microscopic autoradiography. *J. Pharmacol. Toxicol. Methods 51*, 25−40.

(23) Stumpf, W. E. (1995) Vitamin D sites and mechanisms of action: a histochemical perspective. Reflections on the utility of autoradiography and cytopharmacology for drug targeting. *Histochem. Cell Biol. 104*, 417−427.

(24) Yamada, S., Kubota, R., Kubota, K., Ishiwata, K., and Ido, T. (1990) Localization of [^{18}F]fluorodeoxyglucose in mouse brain neurons with micro-autoradiography. *Neurosci. Lett. 120*, 191−193.

(25) Miller, T. R., Milicic, I., Bauch, J., Du, J., Surber, B., Browman, K. E., Marsh, K., Cowart, M., Brioni, J. D., and Esbenshade, T. A. (2009) Use of the H3 receptor antagonist radioligand [^{3}H]-A-349821 to reveal *in vivo* receptor occupancy of cognition enhancing H3 receptor antagonists. *Br. J. Pharmacol. 157*, 139−49.

(26) Codd, E. E., Ma, J., Zhang, S. P., Stone, D. J., Jr., Colburn, R. W., Brandt, M. R., Chevalier, K. M., Zhu, Y., Peng, S., Cai, C., Acton, P. D., and Flores, C. M. (2010) *Ex vivo* delta opioid receptor autoradiography: CNS receptor occupancy of two novel compounds over their antihyperalgesic dose range. *Pharmacol., Biochem. Behav. 96*, 130−5.

(27) Stumpf, W. E. (2005) Drug localization and targeting with receptor microscopic autoradiography. *J. Pharmacol. Toxicol. Methods 51*, 25−40.

(28) Rodríguez-Puertas, R., Pascual, J., Vilaró, T., and Pazos, A. (1997) Autoradiographic distribution of M1, M2, M3, and M4 muscarinic receptor subtypes in Alzheimer's disease. *Synapse 26*, 341−50.

(29) Rodríguez-Puertas, R., Nilsson, S., Pascual, J., Pazos, A., and Hökfelt, T. (1997) ^{125}I-galanin binding sites in Alzheimer's disease: increases in hippocampal subfields and a decrease in the caudate nucleus. *J. Neurochem. 68*, 1106−13.

(30) Gonzalez, S., Fernandez-Ruiz, J., Sparpaglione, V., Parolaro, D., and Ramos, J. A. (2002) Chronic exposure to morphine, cocaine or ethanol in rats produced different effects in brain cannabinoid CB_1 receptor binding and mRNA levels. *Drug Alcohol Depend. 66*, 77−84.

(31) Gillberg, P. G., Nordberg, A., and Aquilonius, S. M. (1984) Muscarinic binding sites in small homogenates and in autoradiographic sections from rat and human spinal cord. *Brain Res. 300*, 327−33.

(32) Fang, Y., Frutos, A. G., and Lahiri, J. (2002) Membrane protein microarrays. *J. Am. Chem. Soc. 124*, 2394−5.

(33) Fang, Y., Lahiri, J., and Picard, L. (2003) G protein-coupled receptor microarrays for drug discovery. *Drug Discovery Today 8*, 755−61.

(34) Fang, Y., Frutos, A. G., Jonas, S. J., Kalal, P. J., and Lahiri, J. (2003) Arrays of biological membranes and methods and use thereof, Patent WO 02/092833 A2.

(35) Rodríguez-Puertas, R., Barreda-Gómez, G., Giralt, M. T., and Fernández-Pastor, B. (2007) Method of quantifying the G protein-coupled receptor (GPCR)/G protein coupling using a cell membrane array, Patent WO/2007/012688.

(36) Posner, B., Hong, Y., Benvenuti, E., Potchoiba, M., Nettleton, D., Lui, L., Ferrie, A., Lai, F., Fang, Y., Miret, J., Wielis, C., and Webb, B. (2007) Multiplexing G protein-coupled receptors in microarrays: a radioligand-binding assay. *Anal. Biochem. 365*, 266−73.

(37) Cassel, D., and Selinger, Z. (1976) Cathecolamine-stimulated GTPase activity in turkey erythrocyte membranes. *Biochim. Biophys. Acta 452*, 538−51.

(38) Koski, G., and Klee, W. A. (1981) Opiates inhibit adenylate cyclase by stimulating GTP hydrolysis. *Proc. Natl. Acad. Sci. U.S.A. 78*, 4185−89.

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

(39) Selley, D. E., and Bidlack, J. M. (1992) Effects of beta-endorphin on mu and delta opioid receptor-coupled G protein activity: low-K_m GTPase studies. *J. Pharmacol. Exp. Ther. 263*, 99−104.
(40) Selley, D. E., Breivogel, C. S., and Childers, S. R. (1993) Modification of opioid receptor−G-protein function by low pH pretreatment of membranes from NG108-15 cells: increase in opioid agonist efficacy by decreased inactivation of G-proteins. *Mol. Pharmacol. 44*, 731−41.
(41) Cerione, R. A., Regan, J. W., Nakata, H., Codina, J., Benovic, J. L., Gierschik, P., Somers, R. L., Spiegel, A. M., Birnbaumer, L., Lefkowitz, R. J., and Caron, M. G. (1986) Functional reconstitution of the α2-adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J. Biol. Chem. 261*, 3901−9.
(42) Hilf, G., Gierschik, P., and Jakobs, K. H. (1989) Muscarinic acetylcholine receptor-stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) to guanine nucleotide-binding proteins in cardiac membranes. *Eur. J. Biochem. 186*, 725−31.
(43) Lorenzen, A., Fuss, M., Vogt, H., and Schwabe, U. (1993) Measurement of guanine nucleotide-binding protein activation by A1 adenosine receptor agonists in bovine brain membranes: stimulation of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding. *Mol. Pharmacol. 44*, 115−23.
(44) Selley, D. E., Sim, L. J., Xiao, R., Liu, Q., and Childers, S. R. (1997) mu-Opioid receptor-stimulated guanosine-5′-*O*-(gamma-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. *Mol. Pharmacol. 51*, 87−96.
(45) Arango, V., Ernsberger, P., Reis, D. J., and Mann, J. J. (1990) Demonstration of high- and low-affinity β-adrenergic receptors in slide-mounted sections of rat and human brain. *Brain. Res. 516*, 113−21.
(46) Gehlert, D. R., and Wamsley, J. K. (1986) *In vitro* autoradiographic localization of guanine nucleotide binding sites in sections of rat brain labeled with [^{3}H]guanylyl-59-imidodiphosphate. *Eur. J. Pharmacol. 129*, 169−74.
(47) Yarfitz, S. L., Running Deer, J. L., Froelick, G., Colley, N. J., and Hurley, J. B. (1994) *In situ* assay of light-stimulated G protein activity in *Drosophila* photoreceptor G protein b mutants. *J. Biol. Chem. 269*, 30340−44.
(48) Sim, L. J., Selley, D. E., and Childers, S. R. (1995) *In vitro* autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[gamma-[^{35}S]thio]-triphosphate binding. *Proc. Natl. Acad. Sci. U.S.A. 92*, 7242−6.
(49) Barreda-Gómez, G., Giralt, M. T., and Rodríguez-Puertas, R. (2010) Methods to measure G-protein-coupled receptor activity for the identification of inverse agonists. *Methods Enzymol. 485*, 261−73.
(50) Laitinen, J. T. (1999) Selective detection of adenosine A1 receptor-dependent G-protein activity in basal and stimulated conditions of rat brain [^{35}S]guanosine 5′-(gamma-thio)triphosphate autoradiography. *Neuroscience 90*, 1265−79.
(51) Sternweis, P. C., and Robishaw, J. D. (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem. 259*, 13806−13.
(52) Rodríguez-Puertas, R., González-Maeso, J., Meana, J. J., and Pazos, A. (2000) Autoradiography of receptor-activated G-proteins in post mortem human brain. *Neuroscience 96*, 169−80.
(53) Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E. L. (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol. 14*, 1675−80.
(54) Schweitzer, B., and Kingsmore, S. F. (2002) Measuring proteins on microarrays. *Curr. Opin. Biotechnol. 13*, 14−9.
(55) Love, K. R., and Seeberger, P. H. (2002) Carbohydrate arrays as tools for glycomics. *Angew. Chem., Int. Ed. 41*, 3583−6.
(56) Lam, K. S., and Renil, M. (2002) From combinatorial chemistry to chemical microarray. *Curr. Opin. Chem. Biol. 6*, 353−8.
(57) Wu, R. Z., Bailey, S. N., and Sabatini, D. M. (2002) Cell-biological applications of transfected-cell microarrays. *Trends. Cell. Biol. 12*, 485−8.
(58) Schoenberg Fejzo, M., and Slamon, D. J. (2001) Frozen tumor tissue microarray technology for analysis of tumor RNA, DNA, and proteins. *Am. J. Pathol. 159*, 1645−50.
(59) Deng, Y., Wang, Y., Holtz, B., Li, J., Traaseth, N., Veglia, G., Stottrup, B. J., Elde, R., Pei, D., Guo, A., and Zhu, X. Y. (2008) Fluidic and air-stable supported lipid bilayer and cell-mimicking microarrays. *J. Am. Chem. Soc. 130*, 6267−71.
(60) Rodríguez-Puertas, R., Barreda-Gómez, G., Fernández González, J. A., Astigarraga Arribas, E., Aranzabe García, E., Marcaide Rodríguez, A., and Gómez Plaza, D. (2008) Method for the surface treatment of solid substrates, Patent WO/2008/01239.
(61) Hong, Y., Webb, B. L., Su, H., Mozdy, E. J., Fang, Y., Wu, Q., Liu, L., Beck, J., Ferrie, A. M., Raghavan, S., Mauro, J., Carre, A., Müeller, D., Lai, F., Rasnow, B., Johnson, M., Min, H., Salon, J., and Lahiri, J. (2005) Functional GPCR microarrays. *J. Am. Chem. Soc. 127*, 15350−1.
(62) Khatib-Shahidi, S., Andersson, M., Herman, J. L., Gillespie, T. A., and Caprioli, R. M. (2006) Direct molecular analysis of whole-body animal tissue sections by imaging MALDI mass spectrometry. *Anal. Chem. 78*, 6448−56.
(63) Hillenkamp, F., and Karas, M. (1990) Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol. 193*, 280−95.
(64) Burns, M. S. (1982) Applications of secondary ion mass spectrometry (SIMS) in biological research: a review. *J. Microsc. 127*, 237−58.
(65) Kertesz, V., Van Berkel, G. J., Vavrek, M., Koeplinger, K. A., Schneider, B. B., and Covey, T. R. (2008) Comparison of drug distribution images from whole-body thin tissue sections obtained using desorption electrospray ionization tandem mass spectrometry and autoradiography. *Anal. Chem. 80*, 5168−77.
(66) Stoeckli, M., Chaurand, P., Hallahan, D. E., and Caprioli, R. M. (2001) Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. *Nat. Med. 7*, 493−6.
(67) Groseclose, M. R., Andersson, M., Hardesty, W. M., and Caprioli, R. M. (2007) Identification of proteins directly from tissue: in situ tryptic digestions coupled with imaging mass spectrometry. *J. Mass. Spectrom. 42*, 254−62.
(68) Astigarraga, E., Barreda-Gómez, G., Lombardero, L., Fresnedo, O., Castaño, F., Giralt, M. T., Ochoa, B., Rodríguez-Puertas, R., and Fernández, J. A. (2008) Profiling and imaging of lipids on brain and liver tissue by matrix-assisted laser desorption/ ionization mass spectrometry using 2-mercaptobenzothiazole as a matrix. *Anal. Chem. 80*, 9105−14.
(69) Koizumi, S., Yamamoto, S., Hayasaka, T., Konishi, Y., Yamaguchi-Okada, M., Goto-Inoue, N., Sugiura, Y., Setou, M., and Namba, H. (2010) Imaging mass spectrometry revealed the production of lyso-phosphatidylcholine in the injured ischemic rat brain. *Neuroscience 168*, 219−25.
(70) Trim, P. J., Djidja, M. C., Atkinson, S. J., Oakes, K., Cole, L. M., Anderson, D. M., Hart, P. J., Francese, S., and Clench, M. R. (2010) Introduction of a 20 kHz Nd:YVO4 laser into a hybrid quadrupole time-of-flight mass spectrometer for MALDI-MS imaging. *Anal. Bioanal. Chem. 397*, 3409−19.
(71) Savina, M. R., and Lykke, K. R. (1997) Microscopic chemical imaging with laser desorption mass spectrometry. *Anal. Chem. 69*, 3741−3746.
(72) Claydon, M. A., Davey, S. N., Edwards-Jones, V., and Gordon, D. B. (1996) The rapid identification of intact microorganisms using mass spectrometry. *Nat. Biotechnol. 14*, 1584−6.
(73) Holland, R. D., Wilkes, J. G., Rafii, F., Sutherland, J. B., Persons, C. C., Voorhees, K. J., and Lay, J. O., Jr. (1996) Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rapid Commun. Mass. Spectrom. 10*, 1227−32.
(74) Krishnamurthy, T., Ross, P. L., and Rajamani, U. (1996) Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass. Spectrom. 10*, 883−8.

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373

(75) Todd, P. J., McMahon, J. M., Short, R. T., and McCandlish, C. A. (1997) Organic SIMS of biologic tissue. *Anal. Chem. 69*, 529−535.
(76) Fragu, P., Briançon, C., Fourré, C., Clerc, J., Casiraghi, O., Jeusset, J., Omri, F., and Halpern, S. (1992) SIMS microscopy in the biomedical field. *Biol. Cell. 74*, 5−18.
(77) Casiraghi, O., Lombard, M. N., Halpern, S., and Fragu, P. (1993) Detection on liver tissue sections of S-phase markers in synchronized cycling rat hepatocytes by SIMS microscopy. *Biol. Cell. 79*, 225−9.
(78) Djidja, M. C., Claude, E., Snel, M. F., Francese, S., Scriven, P., Carolan, V., and Clench, M. R. (2010) Novel molecular tumour classification using MALDI-mass spectrometry imaging of tissue micro-array. *Anal. Bioanal. Chem. 397*, 587−601.
(79) Hankin, J. A., Farias, S. E., Barkley, R. M., Heidenreich, K., Frey, L. C., Hamazaki, K., Kim, H. Y., and Murphy, R.C. (2011) MALDI mass spectrometric imaging of lipids in rat brain injury models. *J. Am. Soc. Mass Spectrom. 22*, 1014−21.
(80) Carracedo, A., Geelen, M. J., Diez, M., Hanada, K., Guzman, M., and Velasco, G. (2004) Ceramide sensitizes astrocytes to oxidative stress: protective role of cannabinoids. *Biochem. J. 380*, 435−40.
(81) Sanchez, C., Galve-Roperh, I., Canova, C., Brachet, P., and Guzman, M. (1998) Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Lett. 436*, 6−10.

DOI: 10.1021/cn500281t
ACS Chem. Neurosci. 2015, 6, 362−373