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In vivo autofluorescence spectrofluorometry of central serotonin

Francesco Crespi[a,*], Anna C. Croce[b], Sabrina Fiorani[b], Barbara Masala[b], Christian Heidbreder[a], Giovanni Bottiroli[b]

[a] *Department of Biology, Psychiatry C.E.D.D., GlaxoSmithKline S.p.A., Verona, Italy*
[b] *Institute of Molecular Genetics, Histochemistry and Cytometry Section, National Research Council, Department of Animal Biology, University of Pavia, Piazza Botta, 10, 27100 Pavia; Italy*

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Abstract

The autofluorescence properties of serotonin (5-HT) were investigated by light spectrofluorometry in in vitro, ex vivo and in vivo experiments. Ex vivo samples were prepared from rat brain regions containing serotonin (5-HT) i.e. cortex, striatum, hippocampus. Rats were untreated (controls) or previously submitted to chronic behavioural or pharmacological treatments known to affect endogenous 5-HT levels. Autofluorescence analysis (excitation: 366 nm) on hippocampus homogenates supplied with exogenous 5-HT revealed spectral alterations attributable to changes of endogenous 5-HT levels. In vivo, real time fluorescence studies were performed via a 50 μm diameter optic fiber probe stereotaxically implanted into selected brain areas of anaesthetised rats treated with fluoxetine or 5-OH-tryptophan.

All autofluorescence data were consistent with those obtained in parallel experiments performed with ex vivo or in vivo voltammetry, confirming that auto-fluorescence spectroscopy is a suitable technique for the direct assessment of fluorescent neurotransmitters. This is a reliable evidence of the in vivo application of spectroscopy together with optic fiber probe for in vivo, in situ and real time measurement of 5-HT in discrete brain areas.

Keywords: Serotonin; Autofluorescence; In vivo spectroscopy; Optic-fibre probe; Hippocampus; Rat brain; Voltammetry

1. Introduction

One of the major challenges in the neuroscience studies is to relate neurotransmitter functions to behaviour in freely moving animals. Recently, in vivo voltammetry and intracerebral microdialysis have allowed reliable measurements of extracellular brain amines and related metabolites as well as neuropeptides. However, both methodologies are based on the use of probes, such as carbon-based microelectrodes or collecting probes, respectively, which have to be inserted into the brain via microsurgery. Thus, both techniques are invasive.

A new approach for the selective, rapid (millisec) and most importantly non invasive detection of neurotransmitters could be based on the analysis of their light (laser)-induced fluorescence emission (LIFE or autofluorescence).

It is well known that most biological compounds when stimulated with suitable excitation light can give rise to a fluorescence emission in the absence of exogenous fluorophores (Aubin, 1979; Rost, 1995). This approach would allow each compound to be analysed in real time in three successive "fields": UV, visible and IR. It would permit the definition of a specific identifying marker for each compound based on its reaction to the different wavelengths applied. This property has been exploited to develop techniques for wide diagnostic applications, especially in the field of oncology (Wagnieres et al., 1998; Baraga et al., 1990; Bottiroli et al., 1995; Andersson-Engels et al., 1997; Holz et al., 1997; Marchesini et al., 1994; Sato et al., 2001; Ramanujam et al., 1996).

For what concern neurotransmitters, serotonin (5-HT) is known to act as an endogenous fluorophore. Absorption and emission bands in the UV region at 290 and 340 nm, respectively, are generally described (Udenfriend 1962). In 1990

* Corresponding author.

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Crespi was reporting that fluorescence analysis could allow a selective measurement of 5-HT versus its metabolite 5-OH-indoleacetic acid (5HIAA) (Crespi, 1990). Thus the near-UV–vis fluorescence microscopy could be in principle considered for the non-invasive analysis of endogenous 5-HT.

However, in the view of the development of an autofluorescence-based technique, the 5-HT spectroscopic properties deserve some comments. In the bulk tissue, which behaves as a turbid medium, the presence of multiple endogenous fluorophores acting as scatterers and absorbers can give rise to a broad band attenuation, and affect both excitation and emission light (Ramanujam et al., 1996). The shorter is the excitation wavelength and more severe are the effects. Depending on the spectral regions interested by the fluorescence properties of a given compound, vantages and disadvantages can arise for the measurements performed in situ. As to the depth of light penetration, for example, the reduced penetrability of the light in the UV interval can be favourably exploited when the diagnostic information comes from changes in defined layers of the tissue (Marchesini et al., 1994). However, more often, effects such as competitive absorption and spectral distorsion adversely affect the measurements performed.

To circumvent these restrictions, two strategies were considered as to 5-HT evaluation is concerned:

(1) providing the excitation energy corresponding to this UV region by means of the sophisticated techniques of multiple-photon excitation, and
(2) moving towards longer excitation wavelengths, exploiting the fact that, even in the case of markedly low extinction coefficients, the benefits associated with decreased light scattering and competitive absorption at longer wavelengths should compensate the poor absorption properties of these transitions.

The former technique allowed, for example, the measurement of 5-HT distribution in in vivo cell preparations (Maiti et al., 1997) and the in situ imaging of changes in oxidation of indoleamines, known to lead to longer wavelength emission (Zipfel et al., 2003). The latter technique, despite weak signals, was successfully applied in the near-UV–vis fluorescence microscopy to the in situ imaging study of the uptake and depletion of neurotransmitters in astrocytes (Tan et al., 1995, 1997; Yeung, 1999). The excitation and emission of 5-HT at longer wavelengths have been recently characterized in more details (Crespi et al., 2004). 5-HT in solution showed minor absorption bands in the 340–590 nm spectral region, and an excitation band in the 310–370 nm region, particularly evident for high 5-HT concentrations. Excitation at 366 nm, in turn, showed a broad emission band in the 390–540 nm region. The same excitation performed on ex vivo samples (hippocampal tissue slices and homogenate) revealed spectral changes attributable to the increase in the amount of 5-HT supplied as exogenous compound or induced in the rats by specific treatments. These data were supported by parallel experiments using conventional voltammetry (Crespi et al., 2004). All these features supported the development of conventional fluorometric techniques to monitor 5-HT levels directly in biological tissues.

In the present work, both ex vivo and in vivo spectrofluorometric studies were performed in discrete rat brain regions such as the hippocampus, striatum and cortex. The animals were previously submitted to pharmacological or behavioural treatments that are known to affect 5-HT levels (Blier and de Montigny, 1994; Kreiss and Lucki, 1995; Wong et al., 1995; Thorre et al., 1997).

The original application of spectroscopy together with optic fibre probes for in vivo, in situ and real time direct measurement of serotonin in discrete brain areas of anaesthetised rats was assessed. Parallel studies using conventional ex vivo and in vivo voltammetric methodologies for the selective measurement of 5-HT (Crespi and Pietra, 1997; Crespi, 2002) were also performed to validate the fluorometric results.

2. Methods

2.1. Chemicals

5-HT and the other chemicals tested in solution were dissolved in PBS at a concentration of 1×10^{-2} M (stock solution), and diluted in PBS or in the homogenate (5-HT) to the final concentrations. For pharmacological treatments, 5-hydroxytryptophane (5-HTP) and fluoxetine were dissolved in saline (NaCl 0.9%) at the predefined concentration before administration. All the chemicals were purchased from Sigma Chem. Co., MO, USA.

2.2. Animals

Naive adult male CD rats (250–280 g, Charles River, Calco, Co., Italy) were used. Experimental procedures were in complete accordance with the guidelines of the "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1985) as well as with the regulation of the Italian laws. All experiments were pre-reviewed and consented by a local animal care committee.

2.3. Treatments

2.3.1. Restraint stress

Rats were immobilised in a cylindrical plastic rodent restrainer for 6 h (between 10:30 and 16:30 h) per day over 4 consecutive days. Unstressed rats (control group) were not subjected to stress, but were accustomed to handling 5 min every day for 4 days. The animals were sacrificed just after the last immobilisation period. Each brain was then rapidly removed, the hippocampus was dissected and immediately homogenised in lysis buffer [5 mM Tris–HCl, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM pepstatin A, 1 mM leupetin, 1 mM aprotinin, pH 8.0]. The homogenate was then processed for autofluorescence spec-

trofluorometry and concomitant differential pulse voltammetric (DPV) analyses of 5-HT levels.

2.3.2. Pharmacological treatments

Rats were treated with fluoxetine either chronically (10 mg/kg daily/12 days, $n = 3$) or acutely (10 mg/kg i.p., $n = 3$). An additional group of three rats were acutely treated with 5-hydroxytryptophane (5-HTP, 10 mg/kg i.p). Control rats received vehicle (NaCl 0.9%, 1 ml/kg i.p., $n = 3$).

2.4. Instrumentation for spectral analysis

2.4.1. Absorption measurements

Absorption measurements were performed by means of the Spectronic Genesys 2 (Spectronic Instruments Inc., Rochester, NY) spectrophotometer with 2 nm spectral bandwidth. Measurements were performed in saline or PBS solutions containing serotonin, noradrenaline, dopamine, GABA, substance P or metabolites such as MOPEG, DOPAC, and nitrites (the concentration tested were ranging from 1 μM up to 1 mM).

2.4.2. Fluorescence measurements

Excitation spectra of tissue homogenates were recorded by means of a spectrofluorometer (model SP-2; Applied Photophysics, London, UK), equipped with a single photon counting system (EG&G-Ortec, Oak Ridge, TN). Excitation spectra were corrected for the wavelength-dependent intensity of the light source by means of a quantum counter (4 g/l Rhodamine B in ethylene glycol).

Autofluorescence emission spectra of tissue homogenates were recorded under epi-illumination conditions by means of a Leitz microspectrograph (Wetzlar, Germany) equipped with an Optical Multichannel Analyzer (OMA, EG&G, Princeton Applied Research, Princeton, NJ), mounting a Jarrell-Ash Monospec 27 spectrograph (Allied Analytical System, Waltham, MA; mod. 82-499, H150 g/mm grating), with a 512-element intensified diode array detector (mod. 1420/512). A 100 W Hg lamp (Osram, Berlin, Germany), combined with KG1 and BG38 anti-thermal filters was used as the excitation source. The excitation light was selected by means of a 366 nm interference filter ($T\% = 25$), and a 390 nm dichroic mirror ($T\%_{366} < 2$). Emission spectra were recorded in the 420–680 nm range. Each spectral acquisition lasted for 10 sequential scans of 200 ms each, for a total measuring time of 2 s. The contributions of both the dark current (instrumental current noise) and the fluorescence of the optics were recorded before each sample measurement and subtracted on-line by the measuring system.

2.5. Measurements in tissue homogenate

The ex vivo spectrofluorometric properties of the homogenate of hippocampal tissue collected from untreated rats were analyzed before and after addition of exogenous 5-HT at various concentrations.

The homogenates of hippocampus, striatum and cortex obtained from untreated (control) and stressed or fluoxetine-treated rats were submitted to both spectrofluorometric studies and concomitant differential pulse voltammetry (DPV) analyses of 5-HT levels. Autofluorescence analysis was performed under epi-illumination conditions by means of the above-described Leitz microspectrograph–OMA system. A drop of homogenate was deposited in the wall of a microscope slide, and the measurement area was selected by means of a field iris diaphragm, ensuring that the homogenate volume involved in the measurement was constant. Alternatively, spectra were recorded directly by immersing the optic fiber probe into the homogenate.

In particular, the spectral profile of 5-HT could be obtained as follow:

(i) the emission spectra in the sample(s) under analysis are monitored before any addition, they are considered as "control spectra"
(ii) the emission spectra in the sample(s) under analysis are monitored following addition of exogenous 5-HT, they are considered as "5-HT spectra"
(iii) subtraction of "control spectra" from the "5-HT spectra".

2.6. In vivo measurements

Anaesthetised (urethane 1.5 mg/kg i.p.) rats were held in a stereotaxic frame (David Kopf) to perform micro-surgery under light microscopy in order to insert the microbiosensor into either the striatum, frontal cortex or hippocampus. This was performed according to the following coordinates: striatum AP 1.0, ML 2.6, DV 5.0; hippocampus AP 4.8, ML 3.8, DV 3.4; frontal cortex AP 2.2, ML 0.5, DV 5.0 mm from bregma (Paxinos and Watson, 1986), respectively. Two types of micro-biosensors were used: the 30 μm diameter carbon fiber micro-electrode (mCFE) used for DPV measurements of 5-HT levels, or the 50 μm diameter optic fiber probe described above and used for the ex vivo and in vivo studies. The low diameter of each biosensor allowed its accurate insertion into discrete brain regions with minor tissue lesions as described previously (Crespi et al., 1995).

The 50 μm diameter optic fiber probe, used to deliver the excitation light and collect the autofluorescence emission for the in vivo spectrofluorometric studies, was coupled to the microspectrograph–OMA apparatus by means of a 10× Leitz objective (NA 0.25). The scheme of the instrumentation arrangement is shown in Fig. 1.

Fluoxetine (10 mg/kg i.p.) or 5-HTP (10 mg/kg i.p.) was then administered according to the conditions described above (see Section 2.3.2). Fluorescence (LIFE) signals or DPV scans were measured every 5 min. Changes in the emission amplitude or in the level of the DPV signal were evaluated with respect to either baseline (control period measurement) or vehicle treatment.

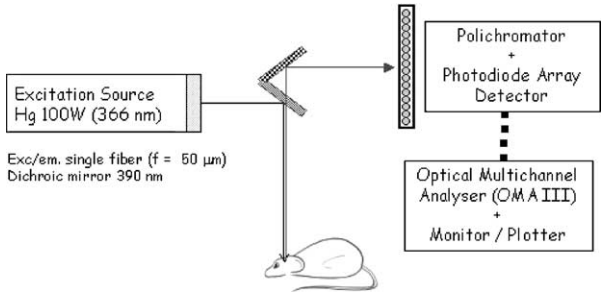

Fig. 1. Scheme of the experimental apparatus used for in vivo measurements. The figure represents the setup of the single optical fibre to be inserted in the rat brain and the dichroic mirror to deliver excitation light and to collect the fluorescence emission.

2.7. Data analysis

Repeated measures analysis of variance or univariate test of significance over parameterized model (type III decomposition, Statistica software, Release 6) as well as paired or unpaired *t*-test have been applied.

3. Results

3.1. Spectrofluorometric characterisation of pure compound in aqueous solution and in tissue homogenate

As already described in detail (Crespi et al., 2004), 5-HT, as a pure compound in solution, exhibits two main absorption bands at about 280 and 300 nm, with minor absorption bands in the 330–390 nm, 450–490 nm, and 510–580 nm regions. At very high concentrations (i.e. 1×10^{-2} M), 5-HT also exhibited an excitation band centred at about 340 nm, which became appreciable at the emission of 460 nm; the emission spectrum recorded under excitation at 366 nm consisted of a broad emission in the 390–540 nm range.

Spectrofluorometric studies performed on hippocampus homogenate to which was added with exogenous 5-HT revealed a spectrum with two excitation bands in the 340–390 nm region when substracting the "control" spectrum obtained before the very addition of 5-HT (Fig. 2A). Under excitation at 366 nm the homogenate did show a main emission band located at about 440 nm. Addition of exogenous 5-HT at concentrations ranging from 5×10^{-6} up to 1×10^{-3} M to the homogenate induced an increase in the emission amplitude, which was quite doubled at the 1×10^{-4} M 5-HT concentration (Fig. 2B).

As to the spectral shape, exogenous 5-HT resulted in the appearance of emission bands in the 450–470 nm and the 520–620 nm regions (Fig. 3A). Again this was evidenced after the subtraction of the spectrum of homogenated before addition of 5-HT from the spectrum obtained after addition of 5-HT (see Fig. 2A).

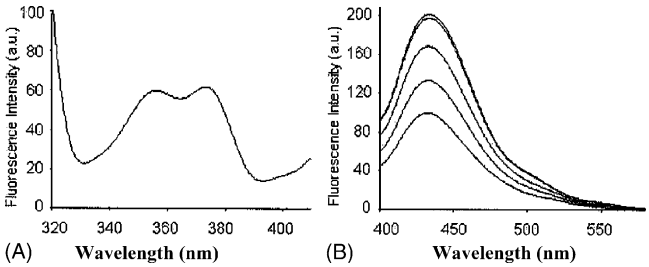

Fig. 2. (A) Excitation spectrum obtained in the rat hippocampus homogenate following addition of exogenous 5-HT 1×10^{-4} M and after subtraction of the initial spectrum recorded in the untreated hippocampus homogenate (0.5 mg proteins/ml). (B) Trend of the emission spectra (excitation 366 nm) monitored in the hippocampus homogenate of untreated rat following addition of exogenous 5-HT at concentrations 5×10^{-6}, 5×10^{-5}, 5×10^{-4} and 1×10^{-3} M, respectively. Lowest curve refers to the homogenate before 5-HT addition.

3.2. Ex vivo spectrofluorometric and voltammetric studies

Fluoxetine resulted in a marked increase in the relative contribution of different emission bands in the 450–470 nm and 520–620 nm emission regions. This was well evidenced

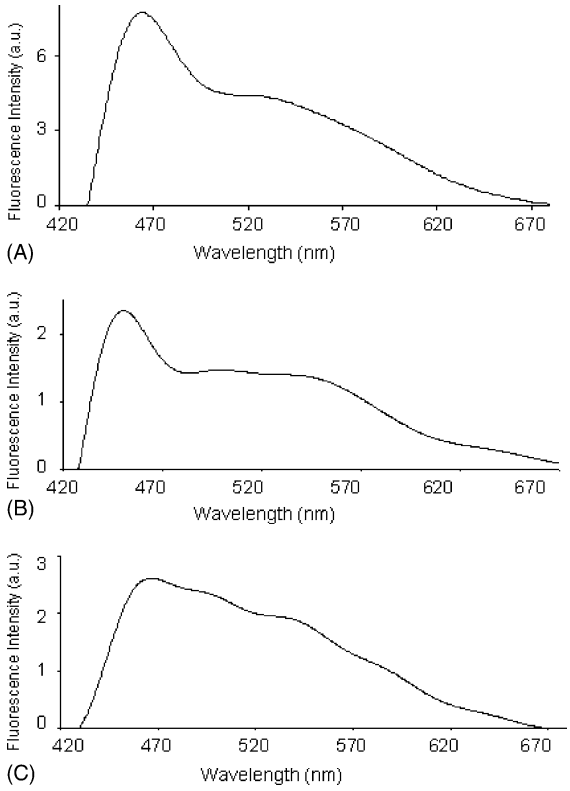

Fig. 3. Representative example of emission spectra recorded in homogenates of: hippocampus (A, B) or striatum (C), following the addition of exogenous 5-HT 5×10^{-5} M (A) or fluoxetine treatment (10 mg/kg i.p.) (B, C) after subtraction of related control spectra. The different features in the bands of the three spectra could be ascribed to the presence of derivatives of 5-HT differing in their oxidation and oligomerisation degrees (Ramanujam et al., 1996).

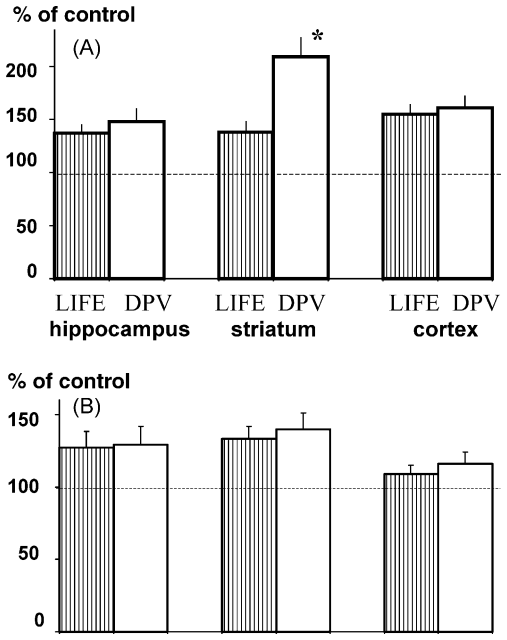

Fig. 4. Concomitant fluorescence intensity (I.F. at 420–470 nm range) and DPV levels of endogenous 5-HT measured in homogenates from the hippocampus, striatum or cortex obtained from rats submitted to chronic fluoxetine (A) or chronic stress (B) treatments. Data are expressed in percent of values from control homogenates.

after subtracting the spectra of the control rats from those of the treated ones, similarly to what obtained when comparing the spectrum obtained in homogenates added with exogenous 5-HT versus untreated homogenates (Fig. 3, A–C).

Spectrofluorometric analysis performed on homogenates of hippocampus, striatum or cortex obtained from rats submitted to chronic treatment with fluoxetine showed an enhancement of the whole autofluorescence emission spectrum when compared to the control values, which was particularly marked in the case of hippocampus and striatum.

Results from parallel LIFE and DPVoltammetric experiments in homogenates from the hippocampus, striatum and cortex of rats submitted to chronic fluoxetine or chronic stress are presented in Fig. 4A–B. Both methodologies were monitoring comparable significant ($p < 0.05$) increases of LIFE and 5-HT signals versus control values (expressed as 100%), respectively. Overall, no statistical differences were obtained when comparing DPV results versus LIFE data in each of the three brain regions studied either using paired or unpaired *t*-test or univariate test of significance over parameterised model. This with the exception of a significant difference observed for striatum DPV data versus LIFE studies in the fluoxetine treated group ($F(1.1) = 39.5$, $p < 0.01$) (Repeated Measures Analysis of Variance).

3.3. *In vivo spectrofluorometric and voltammetric studies*

The brain tissue depth probed at 366 nm was estimated by measuring the fluorescence intensity as a function of tissue thickness, according to Gmitro et al. (1988). Preliminary results on ex vivo rat brain tissue samples whose thickness was varied by stacking serial sections as already described (Croce et al., 1999, 2003) gave a $\mu = [\mu_{\text{eff}}\ (\lambda_{\text{exc}}) + \mu_{\text{eff}}\ (\lambda_{\text{em}})] = 47–49\ \text{cm}^{-1}$. μ_{eff} is the effective optical attenuation coefficient at the excitation and emission wavelengths and represents a combination of absorption and scattering effects for light propagation in tissue. This μ value implies that $(1-e^2)$ or 86% of the total fluorescence signal originates from tissue within a thickness of about 400–420 μm. In other words, the above-described experimental in vivo set-up allows to probe a tissue thickness of about 400–420 μm.

In rats treated acutely with 5-HTP the fluorescence signal increased significantly in hippocampus or in striatum by approximately 18 ± 4% ($F(1.7) = 5.703$ $p < 0.001$) or 24 ± 6% ($F(1.3) = 5.169$ $p < 0.05$) 30 or 40 min after administration, respectively (see Fig. 5 top). In rats administered acutely with fluoxetine (10 mg/kg i.p.) an increase in the autofluorescence

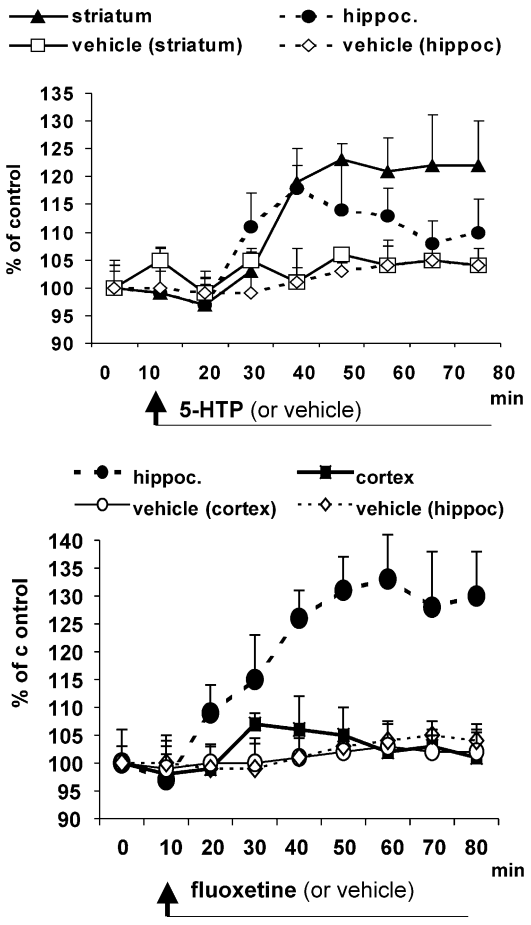

Fig. 5. Top: Time course of the autofluorescence signal recorded in the striatum or in the hippocampus of anaesthetised rats treated with 5-HTP (10 mg/kg i.p. -closed symbols) or vehicle (2 ml/kg NaCl 0.9% -open symbols). Bottom: Time course of the autofluorescence signal recorded in the cortex or in the hippocampus of anaesthetised rats treated with fluoxetine (10 mg/kg i.p. -closed symbols) or vehicle (2 ml/kg NaCl 0.9% - open symbols). $N = 3$ each region and each treatment, data are expressed as percent of control (pre-treatment) values, mean ± S.D.

signal of approximately 33 ± 6% ($F(1.7) = 11.526$ $p < 0.05$) was measured in the hippocampus 50 min post-injection. In the cortex, the increase in the LIFE signal was less important (see Fig. 5 bottom). No changes in LIFE signal were observed following vehicle treatment. Similar data were observed in parallel experiments performed with DPVoltammetry in these rats. For example, a significant increase in extracellular levels of 5-HT by 36 ± 8% in striatum and 41 ± 6% in hippocampus of pre-injection value 40 min after either systemic 5-HTP ($F(1.2) = 17.367$ $p < 0.01$) or fluoxetine ($F(1.4) = 15.0$ $p < 0.01$) treatment, respectively.

In addition, the in vivo LIFE data show a significant difference between the response to fluoxetine in the hippocampus vs. cortex ($F(1.7) = 11.793$ $p < 0.05$: Repeated Measures Analysis of Variance over-parameterized type III). This was not revealed by the homogenate results where both hippocampus and cortex gave similar readouts.

4. Discussion

Spectroscopy is a methodology allowing analysis of compounds in real time in three successive "fields": UV, visible and IR. This allows the definition of a specific identifying "marker" for each compound tested based on its reaction to the various wavelengths applied. While deep UV implies energy that could induce modification of the compound monitored, near UV, visible and IR deal with the natural fluorescence of chemicals without interfering with their structure. This is also an advantage compared with other in vivo methodologies such as voltammetry, where chemicals are oxidised and/or reduced, or versus microdialysis, where there is a modification in the concentration of chemicals and the development of a concentration gradient.

5-HT is known to act as an endogenous fluorophore and the spectral regions generally reported for 5-HT absorption and emission bands are 290 and 340 nm, respectively (Udenfriend, 1962). This is a very short-wavelength spectral region, a condition that, with reference to the optical properties of biological tissues, may limit in situ measurements. To overcome this limitation, 5-HT-autofluorescence properties in longer wavelength spectral regions have been investigated (Tan et al., 1995; Crespi et al., 2004). The benefits associated with decreased light scattering and competitive absorption at longer wavelengths can offset the poor absorption properties at these wavelengths. In aqueous solution 5-HT exhibits selective absorption and fluorescence properties in spectral ranges that can be exploited for conventional micro-spectrofluorometry, namely excitation at 366 nm and emission at wavelengths longer than 400 nm. In addition, the fluorescence emission of 5-HT increases in a low polarity medium such as dioxane (Crespi et al., 2004). This suggests that the possible interaction of the compound with low polarity compounds present in the biological substrate (i.e. proteins, lipids) can favour the detection of 5-HT. The ability to measure endogenous 5-HT fluorescence in a biological environment was supported by changes in fluorescence emission signals recorded in homogenate preparations following the addition of exogenous 5-HT. In ex vivo studies the addition of the pure compound resulted mainly in changes in the emission amplitude, with minor alterations of the spectral shape. Furthermore, the changes in the autofluorescence signal subsequent to the different treatments are related with the expected pharmacological effects. Overall, the data gathered from the behavioural (restraint stress) and pharmacological studies evidence a direct relationship between spectral changes (LIFE data) and the concomitantly measured voltammetric 5-HT signal.

These concomitant experiments were performed in three different brain areas in order to compare-verify the "parallel" answer of the two methodologies. It appeared that the only exception is the non-significant change of LIFE data collected in the striatum of rats treated with fluoxetine versus significant changes of voltammetric data. This could be related to the histologically different structure of such region (the tissue here does have a large amount of myelinated fibers) that may influence the "reading" of the optic probe or/and to the small number of samples tested and therefore should be confirmed in further studies. Nonetheless, the ex vivo data indicate that the fluorescence characteristics of 5-HT permit its detection in biological tissues by LIFE analysis. Consequently, another key outcome of this work is the demonstration that it is possible to use optic fibre probes for ex vivo investigations of endogenous chemicals acting as fluorophores.

The third and possibly the most exciting finding is the observation that the optic fibre probe could also be used for the direct in vivo monitoring of the changes of endogenous 5-HT levels in discrete brain regions of rodents following behavioural and pharmacological treatments. Similar data were indeed collected using in vivo LIFE and in vivo voltammetry.

Finally, LIFE data indicate that the above-described experimental set-up allows to probe the tissue autofluorescence properties and therefore the biochemical nature of the substrate, at depth up to 400–420 μm. This could permit the direct, not invasive analysis of the outer layers of the cerebral cortex. Here beaded serotonin axon terminals are primarily found. This serotonergic innervation of the cortex originates mainly from the raphe dorsalis nucleus (for a review see Heidbreder and Groenewegen, 2003).

Therefore, another advantage of the optic fibre probe is that it could be positioned just outside the brain area to be studied, thus maintaining its physical integrity. Studies examining the feasibility of avoiding penetration of the optic probe into the CNS are currently under investigation. Such studies will provide the basis for the development of a non-invasive, real time diagnostic technique by exploiting the 5-HT absorption properties at longer wavelengths. Excitation in the 550–590 nm range can in fact ensure a light penetration depth up to a few mm, which should allow deeper non-invasive inspection in living tissues.

The potential of the long wavelength excitation approach is at present under investigation.

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