



Review Article

Serotonin, how to find it...

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Abstract. Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter. Biochemically derived from tryptophan, serotonin is primarily found in the gastrointestinal tract, platelets, and in the central nervous system (CNS) of animals, including humans. Discovered and crystallized over sixty years ago, serotonin operates as a short-range neurotransmitter as well as a long-range signalling modulator, with multiple effects on whole organism functions via plasma, platelet and neuroendocrine, gut, adrenal and other peripheral systems across many species. All of the important functions of serotonin in the brain and body were identified over the ensuing years by neurochemical, physiological and pharmacological investigations. Mainly, all these investigations have been performed via invasive methodologies, particularly in the CNS studies. Here we present a rapid overview of such methodological approaches focussing on voltammetry, one of the most recent technical approaches for serotonin analysis in vivo, in situ and in real time. Furthermore, we introduce a late technical evolution in the attempt to obtain in vivo non invasive measurement of brain serotonin.

Abbreviations

Serotonin (5-hydroxytryptamine; 5-HT); central nervous system (CNS); 5-hydroxy-indol acetic acid (5-HIAA); carbon fibre micro electrodes (μ CFE); differential pulse voltammetry (DPV); direct current amperometry (DCA); light-induced fluorescence excitation (L.I.F.E.); Near Infrared Spectroscopy (NIRS); ultraviolet (UV); selective serotonin reductase inhibitor (SSRI)

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1 Introduction

1.1 Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) was initially discovered as a vasoconstrictor substance in blood and later in blood vessel walls, platelets and in enterochromaffine cells of the gastrointestinal system, the lungs and the heart (Vialli et al. 1933; Rapport et al. 1948; Peroutka et al. 1994). More than 50 years ago the chemical structure of 5-HT was identified and synthesised (Twarog et al. 1953). Later, the function of 5-HT as a neurotransmitter in the CNS was proposed (Bogdanski et al. 1956) and has been studied intensively since its identification in the pituitary gland (Hyypa et al. 1973).

In the central nervous system (CNS) serotonin is synthesised in the perikarya of the neuron where the diet amino acid tryptophan is hydroxylated to the 5-HT precursor 5-hydroxytryptophan (5-HTP), which is then decarboxylated to 5-HT (Hamon et al. 1982). To avoid immediate enzymatic oxidation to 5-hydroxy-indol acetic acid (5-HIAA) by monoamine oxidase, 5-HT is contained in neuronal vesicles until it is released into the synaptic cleft. Serotonin then activates either post-synaptic or pre-synaptic receptors or undergoes reuptake via the 5-HT transporter molecule (Sert) into the neuron (Hamon et al. 1982).

Serotonin is involved in regulation of the central neuroendocrine system as well as cognitive functions, mood and basal physiological functions (Van de Kar 1991). Dysfunction of the intra- and interneuronal 5-HT transmitter systems may result in impairment of coping with states of increased stress, cognitive dysfunction and eventually mental diseases (Graeff et al. 1996; Roth et al. 2004). Furthermore, the 5-HT system is involved in regulation of gastrointestinal function and in the development of diseases such as migraine, obesity and nausea

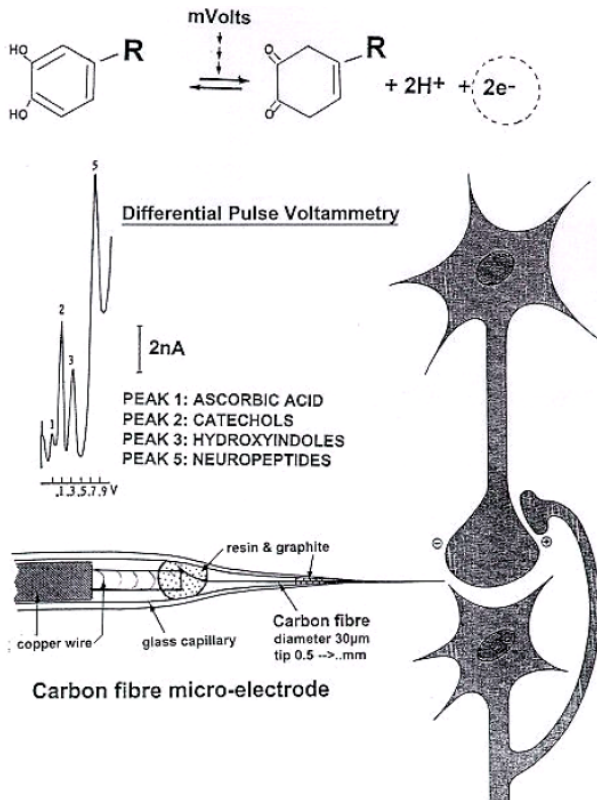


Figure 1: Voltammetry: top: principle, middle: the voltammogram obtained *in vivo* in the CNS when using DPV together with μ CFE; bottom: schematic drawing of the μ CFE and its theoretical positioning within the brain tissue [not to scale].

(Meguid et al. 2000; Saxena 1995).

1.2 5-HT distribution

5-HT distribution in the brain is diffuse and complex. The location of 5-HT containing cell bodies in the CNS was first shown by Dahlstrom et al. (1964) using the fluorescent histochemical technique introduced by Falk et al. (1962). In 1981, an extremely detailed mapping of 5-HT pathways in the CNS was performed using a specific immunohistochemical technique (Steinbusch 1981).

1.3 5-HT cell bodies

The majority of cell bodies lie within the brain stem and mesencephalon in nine groups labelled B1 to B9. Cell bodies B1 to B4 and B6 are located in the medulla. The B1-raphé pallidus, B2-raphé obscurus, B3-raphé magnus and B4/B6-region existing under the 4th ventricle. Group B5-cell bodies lie within the raphé dorsalis. Finally, groups B8 and B9 are located in the mesencephalon and lie within the raphé medianus and lemniscus medialis respectively (Steinbusch 1981). 5-HT cell bodies have also been reported by various authors in the locus caeruleus and sub-caeruleus.

1.4 5-HT pathways

5-HT nerve terminals extend throughout the brain and spinal cord (Steinbusch 1981). Briefly, the 5-HT innervation of the brain can be divided into two main ascending axon bundles termed the medial and lateral ascending pathways (originating from cell bodies in B-5-6-7-8-9) and descending bulbo-spinal neurones (originating from the raphe nuclei of the medulla oblongata). The main brain areas innervated by 5-HT neurones include the thalamus, hypothalamus, subthalamus, olfactory areas, cortex, basal ganglia, septum, hippocampus and substantia nigra.

2 Methods

2.1 5-HT release

In vitro studies of 5-HT release are limited by the rapid re-uptake of released 5-HT, necessitating the presence of an uptake inhibitor in the incubation medium (Blackburn et al. 1967) and can only give an approximation of actual *in vivo* amine levels as the complex cellular and sub-cellular events in tissue cannot be duplicated *in vitro*.

The first notable *in vivo* techniques developed for *in vivo* analysis of 5-HT release are the technique of “push-pull cannulae” (Gaddum, 1961) a perfusion-based technique, as well as “intracranial microdialysis” (Ungerstedt, 1984). These are two similar methods with major limitations, briefly: i] excessive dimension of the probe thus limiting the analysis to large brain areas with tissue damage (Yaksh et al. 1974; Khan et al. 2003); ii] all perfusion techniques make an indirect measurement of release as they measure diffusion and not strictly release, with the chemicals collected being measured “off line”. These methods also have quite a slow response time, 10-30 min is needed to collect a volume of perfusate great enough to allow the detection of chemicals within it. Their detection is thus not performed in “real time” with, in consequence, a poor correlation between changes in their extracellular levels, neuronal activity and behaviour.

An alternative method is voltammetry: in 1924 Heyrovsky found that the current at a mercury electrode was not directly proportional to the applied voltage, but that there was the presence of an extra-current determined by the oxidisable chemicals present in the solution. Such extra-current, that is proportional to the concentration of the compound(s) oxidised and/or reduced, is called polarographic current when obtained at a mercury electrode, and voltammetric current when obtained at all other types of electrode (Adams 1969 a,b). Different types of voltammetric techniques are available, the most common of which are chrono-amperometry linear voltammetry, cyclic voltammetry, and pulse voltamme-

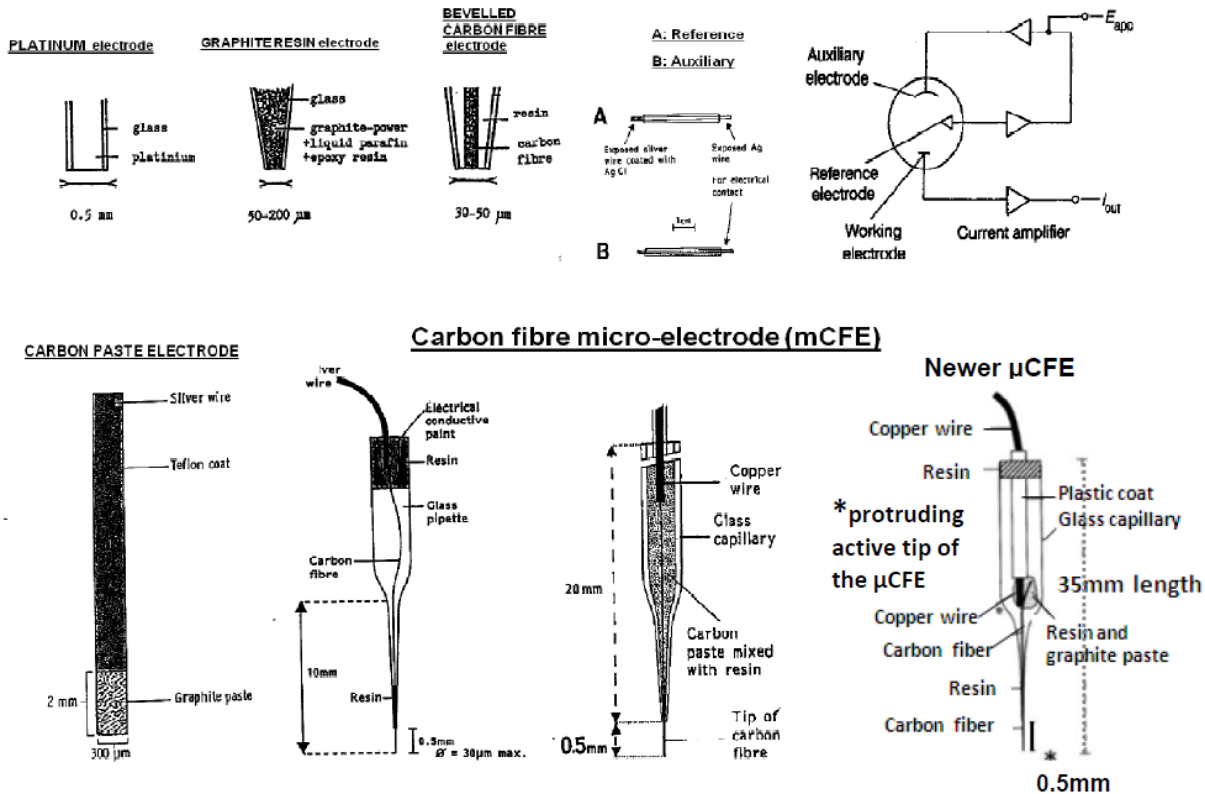


Figure 2: Various types of voltammetric electrodes developed since the 1970s, from the first ones made of platinum wire or teflon coated silver wire to the carbon paste based electrodes and finally to the carbon fibre electrodes (for a review: Stamford et al. 1992).

try. These methodologies are mainly based on the application of a “dynamic” oxidation or oxido-reduction potential and the resulting analysis of electrons “freed” by the chemical(s) under analysis (Fig.(1.1)).

Different types of voltammetric electrodes have been developed since 1969, the most successful type appears to be carbon based electrodes and in particular the carbon fibre micro electrodes (μ CFE) see Fig.(2).

2.2 In vivo voltammetry

An advanced approach to monitoring changes in monoamine release and their metabolism is the technique of in vivo voltammetry using micro biosensors, mainly carbon-fibre micro electrodes (μ CFE) with a 7 to 30 micrometer diameter (see Figs.(1, 2, 3)). The method fulfils many of the criteria required to monitor specific compounds in the extracellular fluid:

I- Measurements can be made in the extracellular fluid of specific large as well as small brain nuclei with minimal damage to the nervous tissue and disturbance to the animal due to the small dimensions of the probe (see Figs.(1, 3)), which can sample an area of approximately $10\text{-}6\text{mm}^3$. Thus there is a clear high anatomical resolution of the site of measurement within discrete brain regions of rodents. Furthermore, no signs of the presence of the micro electrode could be observed in the brain tissue when the histological evaluations correct (or

incorrect) location of its active tip, within the brain region studied, was performed under light microscopy at the end of each experiment when the brain was rapidly removed and sectioned using a cryostat. This indicates that the sole insertion of μ CFE into the brain does not produce tissue lesions detectable by light microscopy. Indeed, a lesion (coagulated brain tissue) was necessary and was obtained at the end of each experiment via application of a direct current through the active tip of the μ CFE to verify such position under light microscopy in brain slices stained using the NISSL solution. II- The method allows rapid, repeated measurements with accurate time resolution in vivo, in situ in real time without recourse to perfusion based techniques or radiolabelled transmitter stores, or the need for sample preparation or chromatographic separation. This is the fundamental difference between voltammetry and perfusion techniques (Crespi et al. 1988; Stamford et al. 1992).

III- In vivo voltammetry can be performed in conscious freely moving animals. This, combined with points I and II, avoids the problems associated with anaesthetics and allows correlations within neuronal activity behaviour. Furthermore, recent improvement in the methodology allow voltammetric analysis in telemetric – wireless conditions allowing electrochemical studies in absolute freely moving conditions (Crespi 2010a) (see Fig.(3)). Indeed, the enhanced telemetric system based

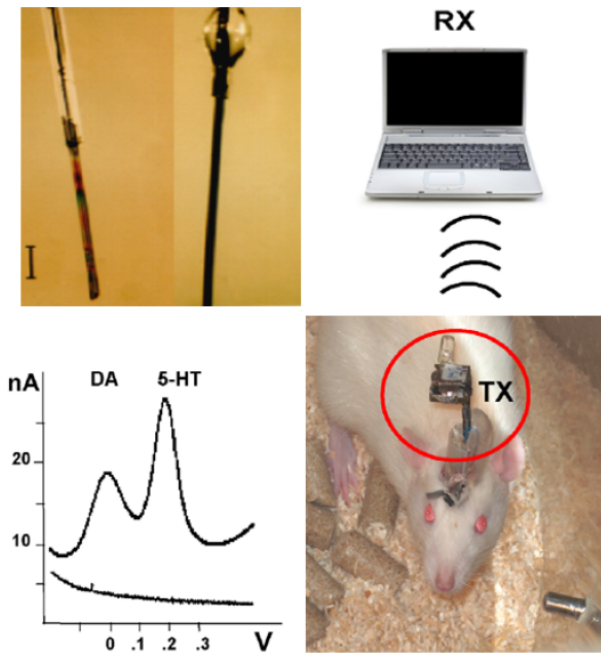


Figure 3: In vivo voltammetry in conscious freely moving conditions
Top left: magnification of the electro-active tip of the μ CFE before (right) and after Nafion[®] coating (left). Bar: 100 μ m.
Bottom right: the behaving freely moving rat with the transmitter unit TX placed upon its head transmitting the signal via an infrared channel to the receiving station (RX). The TX is a miniaturised circuit (dimension of about 0.5cm³) of much reduced weight (5–6g). It is mounted on the animal's head and connected to the voltammetric three-electrode system inserted into the brain. A laptop computer, interfaced to the receiving station, performs the required signal acquisition and analysis (top right).
Bottom left: representative in vitro DPV curves for dopamine 5 μ M and serotonin 5 μ M as measured with Nafion[®]- μ CFE (Crespi et al. 1988). In vivo studies, the tip of the Nafion[®]- μ CFE is inserted in a discrete brain area, i.e. the rat prefrontal cortex as shown by the red arrow in E. [With permission from Biosensors and Bioelectronics].

on either differential pulse voltammetry (DPV) or direct current amperometry (DCA) “diffused” via a single-way infrared (IR) transmission channel is of much reduced dimension and weight. In particular, parallel in vivo experiments in rats prepared for classical wire-connected DCA or for wireless DCA resulted in superimposed data when the 5-HT system in the frontal cortex was challenged with fluoxetine (Crespi 2010a).

2.3 Biogenic amines or metabolites?

As introduced above, voltammetry in association with specifically prepared micro-biosensors detects the cerebral extracellular content of neurotransmitters and respective metabolites.

Pharmacological experiments have indeed demonstrated that ascorbate, noradrenaline and/or dopamine (and its metabolite DOPAC), 5-OH-indoles (i.e. serotonin and its metabolite 5-HIAA) (Crespi 1990; Crespi et al. 1988) uric acid, (Crespi et al. 1983b) homovanillic acid, 3-methoxytyramine (Crespi et al. 1989b) as well as

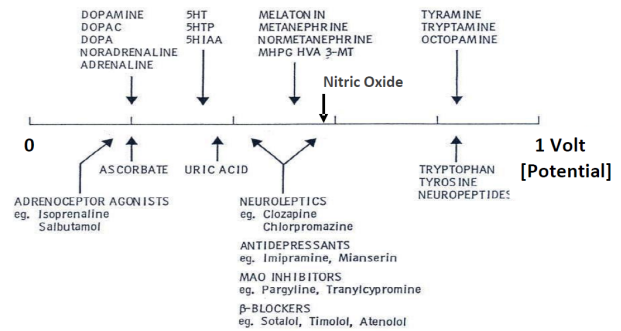


Figure 4: The figure shows different compounds presenting electro-active chemical groups so that they can be measurable with voltammetry.

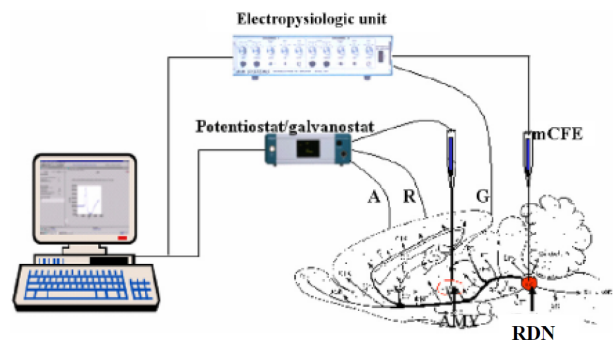


Figure 5: The voltammetric-electrophysiological set up consists of the concomitant use of a potentiostat three-electrode system (A: auxiliary, R: reference and the μ CFE) and an electrophysiological two-electrode unit (G: ground electrode and the μ CFE). Electrodes A, R and G were inserted between the bone and the dura mater through holes (200 μ m diameter) that were drilled in the parietal bone. The μ CFEs were inserted in the specific brain regions studied (i.e. amygdale [AMY] and raphe dorsalis nucleus [RDN], respectively) under light microscopy. All electrodes were connected with electrical wires to the polarograph or the electrophysiological apparatus driven by specific softwares (GPES Ecochemie, The Netherlands or MSD Alpha-Omega, Israel, respectively).

neuropeptides containing electroactive amino acids such as tryptophan, cysteine, tyrosine, (Crespi 1991, Crespi 2011) melatonin and (Crespi 2012; Crespi et al. 1994) nitric oxide (Crespi et al. 2001; Rossetti et al. 2004), can be selectively monitored with this methodology.

At one stage it was thought that in vivo voltammetry could be used to study BIOGENIC AMINES such as DA, 5-HT release directly in situ and in real time (Adams et al. 1978; Marsden et al. 1979). Later on, however, it was determined that the voltammetric signal in vivo was mainly correlated to the oxidation of extracellular metabolites (Crespi et al. 1984) (see Fig.(4)).

The development of a new voltammetric biosensor (the Nafion carbon fibre micro-electrode described in Fig.(3)) made it possible to directly measure the release of 5-HT in vivo (Crespi et al. 1988).

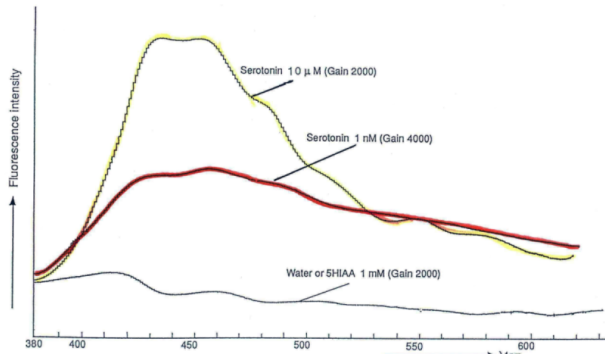


Figure 6: Fluorescence intensity induced by LASER excitation of solutions containing 5-HT nanoM or microM concentrated and 5-HIAA milliM concentrated versus water.

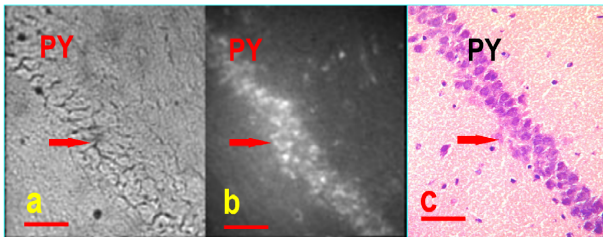


Figure 7: Typical histological pattern of the hippocampus containing the pyramidal cell layer:
 (a) Pyramidal 5-HT cells (PY): non stained section without excitation AND
 (b) Under excitation at 366nm, resulting in a similar pattern as in (c):
 (c) Pyramidal 5-HT cells (PY) stained with hematoxylin eosin, bar=90μm.
 Arrows indicate the same histological position.

2.4 Parallel electrochemical [voltammetric] and electrophysiological in vivo studies

It has also been shown that voltammetric analysis of neurotransmitter release and metabolism can be coupled with analysis of vigilance states via coupling EEG, EOG and EMG recordings with electrochemical [voltammetric] measurement. In particular, this approach has been very valuable for studying the putative relationship between sleep-awake circadian rhythm and serotonergic activity in the raphe regions of rodents; i.e., demonstrating a direct relationship between sleep-awake circadian rhythm and release of 5-HT in the raphe nuclei (Crespi and Jouvet 1982), the brain region involved in the regulation of such sleep-awake circadian rhythm (Jacobs and Azmitia 1992; Ursin 2002).

The feasibility of concomitant in vivo recordings of electrophysiological signals such as cell firing and voltammetric measurements of unstimulated levels of extracellular compounds has also been demonstrated either with these two independent techniques combined in vivo at a single electrode (Crespi 2002) or with parallel recordings in cell bodies; i.e., dorsal raphe nucleus [electrophysiology] and voltammetric recordings in related

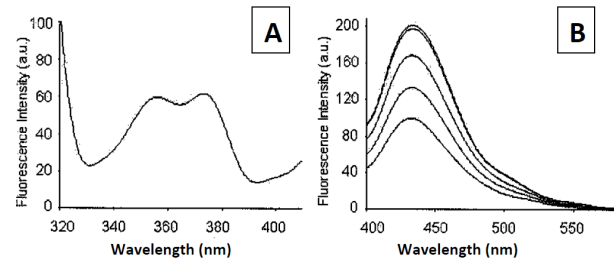


Figure 8: **Left:** Excitation spectrum obtained in the rat hippocampus homogenate following addition of exogenous 5-HT 1×10^{-4} M and after subtraction of the initial spectrum recorded in the untreated hippocampus homogenate (0.5mg proteins/ml). **Right:** Trend of the emission spectra (excitation 366 nm) monitored in the hippocampus homogenate of untreated rat following addition of exogenous 5-HT at concentrations 5×10^{-6} M, 5×10^{-5} M, 5×10^{-4} M and 1×10^{-3} M, respectively. Lowest curve refers to the homogenate before 5-HT addition.

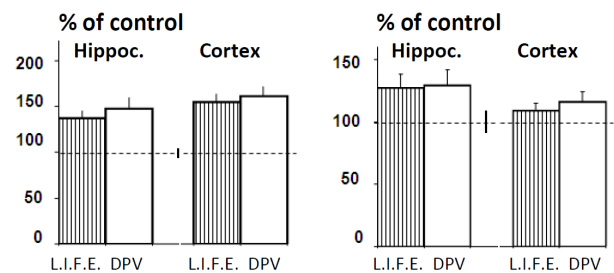


Figure 9: Concomitant fluorescence intensity (L.I.F.E. at 420-470nm range) and DPV levels of endogenous 5-HT measured in homogenates from the hippocampus or cortex obtained from rats submitted to chronic fluoxetine (Left) or chronic stress (Right) treatments. Data are expressed in percent of values from control homogenates. Overall, no statistical differences were obtained when comparing DPV results versus L.I.F.E. data using either paired or unpaired T-Test or univariate test of significance over the parameterised model (for further details see Crespi et al. 2004).

terminal regions or in the amygdala (Crespi 2009)(see Fig.(5)). This verifies the original proposal that a combined treatment with a potassium (SK) channel blocker such as apamin and fluoxetine could overcome the slow onset of the SSRI upon central 5-HT activity that could be related to the slow onset of its therapeutic antidepressant action (Crespi 2010b). Electrochemical and behavioural evidence of a direct relationship between cerebral 5-HT and cytoskeleton in the control of mood was also confirmed (Crespi 2010c). Concomitant behavioural and voltammetric analysis could also be performed, proposing that divergent central serotonergic activities may be responsible for either despair or learning behaviour in intact Wistar or Sprague-Dawley CD rats, respectively (Crespi 2010d)

2.5 Serotonin measurements: from invasive to non – invasive approaches

In 1962, Udenfriend showed that serotonin can act as a fluorophore as its light-absorption and emission properties in aqueous solution occur in the near-UV-visible region.

In 1979, Aubin showed the existence of an auto-

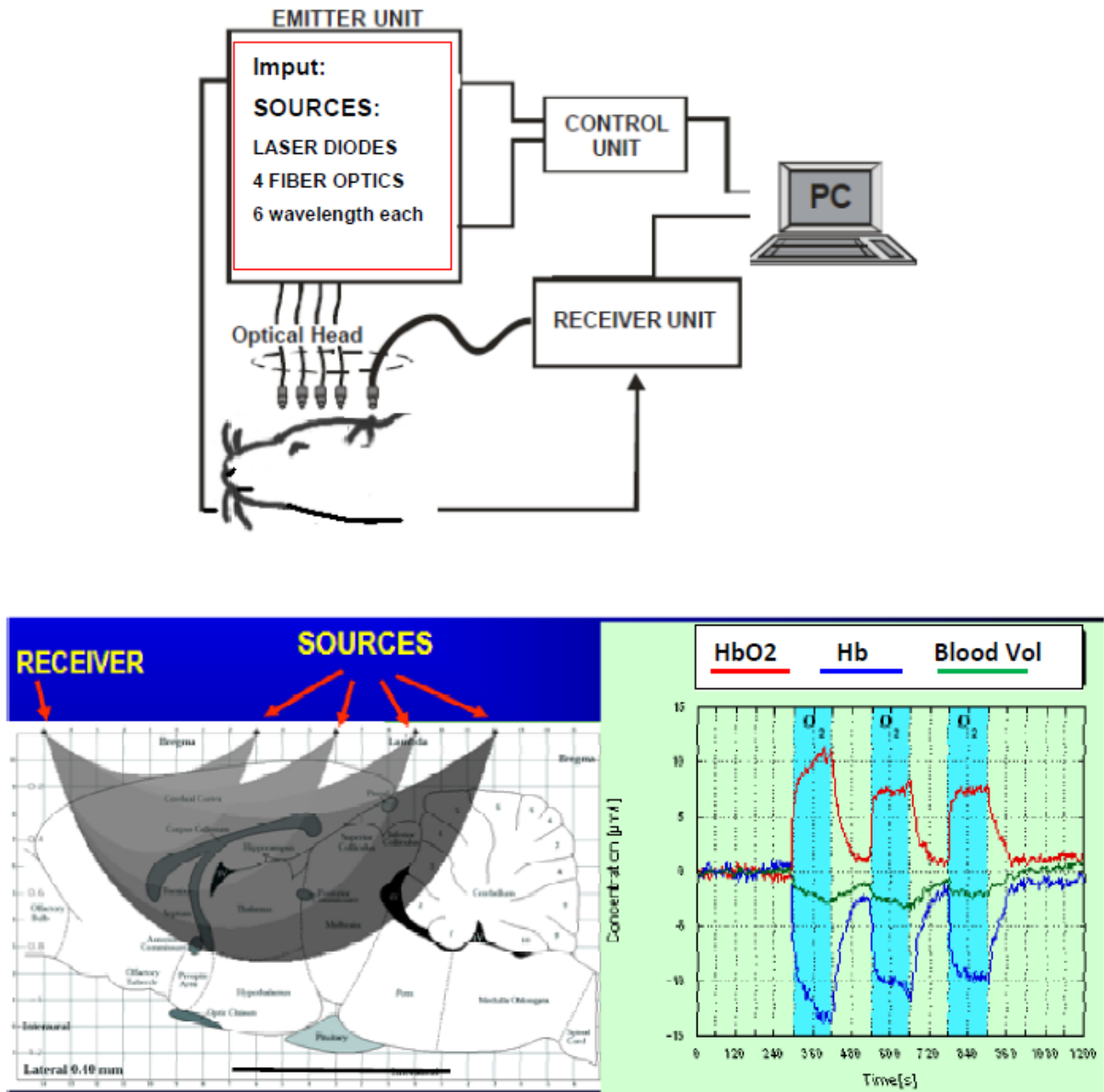


Figure 10: In vivo NIRS: **Top**: block diagram of the NIRS prototype applied to rodents. **Bottom left**: brain areas monitored using four sources and one receiver: computer simulation of photon paths based on photon migration theory. **Bottom right**: changes of physiological parameters following pure oxygen administration (for further details see Crespi 2007).

fluorescence emission in the UV-visible interval when biological tissues were submitted to suitable light stimulating conditions.

Based upon this evidence, in 1990 Crespi had proposed the use of light-induced fluorescence excitation (L.I.F.E.) in the attempt to selectively monitor neurotransmitters based upon analysis of their own fluorescence. This approach demonstrated that the autofluorescence properties of a neurotransmitter such as serotonin can be selectively evaluated (see Figs.(6, 7)). Then L.I.F.E. spectroscopy was performed in ex vivo and in vivo experiments: it was observed that serotonin exhibited the well known excitation and emission bands

in the UV region (i.e. 250-320nm) and also other minor excitation and emission bands in the near UV region (i.e. 450-580nm). Furthermore, spectrofluorimetric measurements under 366nm excitation performed on solutions supplied with serotonin or on brain homogenates obtained from control or specifically treated rats, were consistent with those obtained in parallel experiments using in vitro or ex vivo voltammetry (see Fig.(8)), therefore proposing the application of in vivo L.I.F.E. Indeed, in vivo L.I.F.E. studies performed in situ and in real time by means of 50µm diameter optic fiber stereotaxically implanted in discrete brain areas of anaesthetised rats, again resulted consistent with

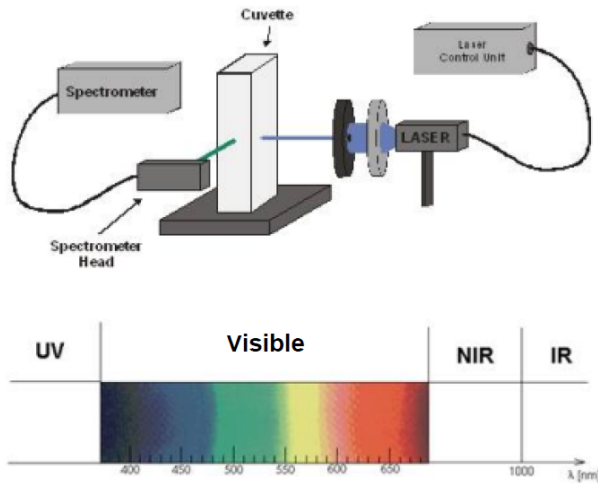


Figure 11: Set-up of instrumentation for in-vitro fluorescence measurements and Laser source spectrum: from near IR to near UV.

parallel in vivo voltammetric experiments performed in rats treated with chemicals selectively affecting the 5-HT system (Crespi et al. 2004) (see Fig.(9)). However, this methodology as well as in vivo voltammetry, remains invasive.

In the attempt to overcome invasiveness, Near Infrared Spectroscopy (NIRS) methodology was applied: by means of prototype instrumentation for analysis in small rodents (Fig.(10)). This technique allows non invasive in vivo preclinical studies of CNS metabolic functions via direct measurement of oxyhaemoglobin and deoxyhaemoglobin (Crespi 2007). In addition, it permits the assessment of real time brain penetration and efficacy of drug treatments (Crespi et al. 2006).

Therefore NIRS permits (as well as MRI) translational strategy from preclinical to clinical investigations.

2.6 Laser source spectrum: from near IR to near ultraviolet (UV)

Based on NIRS (i.e. non-invasive laser based methodology) is the actual attempt to use NON invasive spectroscopy to analyse neurotransmitter in the rat CNS. The technical principle is the same described for the NIRS study of brain metabolism, the main difference related to the source-receiver system needed for detecting natural or induced fluorescence of endogenous chemicals acting as neurotransmitters such as serotonin. For this purpose, other types of sources have been taken into consideration and in particular UV laser sources have been selected such as the Hamamatsu M8903-01 that is a pico-second light pulser with wavelength of 402 nm (near UV wavelength spectrum: 300 to 400nm). It has been combined with the Hamamatsu spectrometer H8353 and a dedicated optical set-up (see Fig.(11)). This source has first been tested using fluoresceine, a compound known to have a spectrum of fluorescence within the green band of the visible wavelength; i.e. 480-560nm.

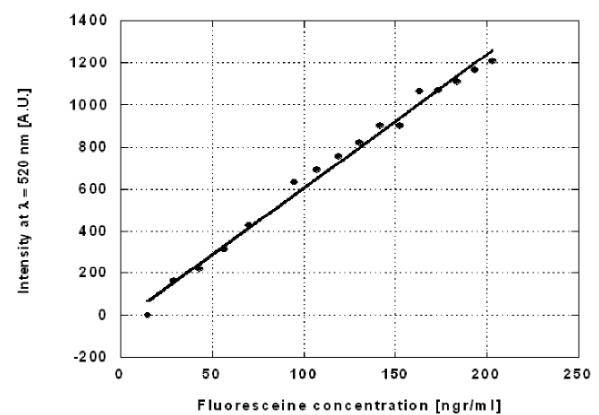
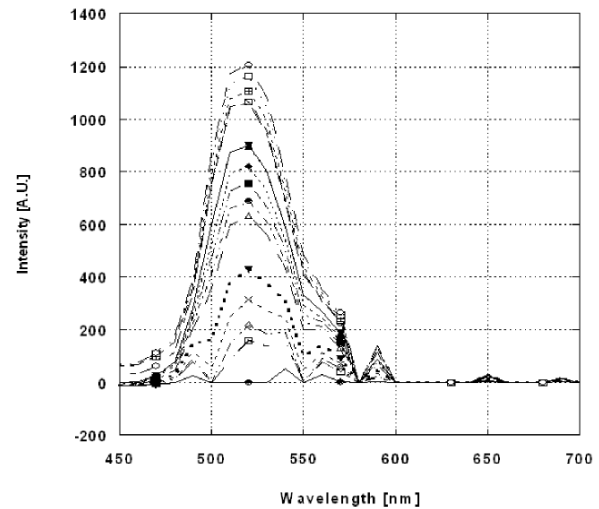


Figure 12: **Top:** Fluoresceine emission spectra obtained with fluoresceine tested at various concentrations: emission intensity peak [arbitrary units, U.A.] is monitored at 520nm;

Bottom: Experimental point of revealed intensity at 520nm as a function of fluoresceine concentration.

To do so the Hamamatsu spectrometer H8353 has been completely characterized using the optical set-up shown in Fig.(11), in particular the cuvette contains a solution with different concentrations of fluoresceine: from 10microM up to 100microM in order to test the linearity of the receiving unit. The output beam of the laser source has been collimated by means of a bi-convex lens with focal length $f=10$ mm. Emission spectra of these fluoresceine concentrations are shown in Fig.(12) TOP, confirming the linearity of the receiving unit. The emission intensity peak was obtained at 520nm and it appeared that the increase in the emission intensity is related to fluoresceine concentration as assessed in Fig.(12) BOTTOM, where the linearity error is 3.19% full-scale.

Based on the L.I.F.E. spectroscopy observations reported above showing that serotonin exhibited also other minor excitation and emission bands in the near UV region, i.e. 450-580nm, experiments were performed in the same conditions described for fluoresceine. The preliminary data gathered, to be confirmed in further

studies, indicate that 5-HT could be also monitored with this set-up within the blue region. Different 5-HT concentrations were tested (1, 10 and 100 µM) and data lead to meaningful differences in the intensity of the fluorescence spectra.

3 Concluding Remarks

In conclusion, non-invasive laser based NEAR UV methodology is introducing very promising results on the attempt to analyse auto-fluorescent neurotransmitters, such as serotonin, using optic fibres in vivo and in non-invasive conditions.

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