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# Determination of NADH in the rat brain during sleep-wake states with an optic fibre sensor and time-resolved fluorescence procedures


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## Abstract

The present paper reports a nanosecond time-resolved fluorescence derived from the cortex and the area of the periaqueductal gray including the nucleus raphe dorsalis (PAG-nRD) in unanaesthetized freely moving rats. The measurements were acquired through a single optic fibre transmitting a subnanosecond nitrogen laser pulse (337 nm, 15 Hz) and collecting the brain fluorescence occurring at 460 nm which might depend on mitochondrial NADH (reduced form of nicotinamide adenine dinucleotide). The fluorometric method was combined with polygraphic recordings, and this procedure allowed us to define, for the first time, variations of the 460 nm signal occurring throughout the sleep-wake cycle. In the PAG-nRD, the signal exhibited moderate heterogeneous variation in amplitude during slow-wave as compared to the waking state. Constant increases were observed during paradoxical sleep as compared to the waking state. For this state of sleep the magnitude of the variations depended on the optic fibre location. In the cortex and during either slow-wave sleep or paradoxical sleep, the signal presented moderate increases which were significant during paradoxical sleep.

The magnitude of the redox variations observed either in the PAG-nRD or in the cortex might be ascribed to the oxidative energy balance which is related to sleep states.

## Keywords

- optic sensor;
- laser;
- NADH;
- brain;
- sleep;
- rat

## Abbreviations

- AcCoA, acetyl coenzyme A;
- ACh, acetylcholine;
- CSF, cerebrospinal fluid;
- EEG, electroencephalogram;
- EMG, electromyogram;
- FOCS, fibre optic chemical sensor;
- 5-HT, serotonin;
- nRD, nucleus raphe dorsalis;
- PAG, periaqueductal gray;
- PDH, pyruvate dehydrogenase;
- PS, paradoxical sleep;
- SWS, slow-wave sleep;
- W, waking state

The use of biochemical sensors or of permeation methods allowing measurement of brain-specific compounds is actually a well established procedure. These tools are suitable for behavioural studies since they are mini-invasive and sufficiently sensitive to detect, for example, extracellular changes occurring in neurochemicals across sleep-wake alternations.9 and 74
Biochemical sensors, through an electrochemical detection, allow quasi-continuous measurements without causing significant disturbances to the brain. Nevertheless, they are only suitable for a limited number of compounds, generally the amines and their metabolites [74], nitric oxide11 and 47 and glucose.[72] Permeation methods allow measurement of a greater variety of substances, but are limited by their anatomical resolution and the time-lag necessary to collect the fractions between successive measurements.[4]
To overcome these difficulties, we introduced an alternative technique using a fibre optic chemical sensor (FOCS), easily implantable in the brain of animals under chronic conditions and allowing fast measurements of the brain chemical contents in combination with sleep polygraphic recordings. Since this approach is applicable to several fields, for clarity's sake we shall review the issues associated with each field.

## 1. Introduction

### 1.1. Technical aspect

The first detailed spectrofluorometric study performed in the brain used the “surface microfluorometry” technique for detection of brain NADH fluorescence. [12] Since then, the authors have also used a Y-shaped optic fibre ($\varphi$=200 $\mu$m) to monitor the NADH fluorescence in the surface of the rat brain. [50] These approaches have allowed the emergence of multiple technical improvements, i.e. laser-induced fluorescence system, micro-optical fibre, fluorescence microscopy, video imagery and time-resolved fluorescence techniques. 22, 32, 40, 50, 66 and 70 Lastly, and in addition to the above aspects, light reflectance has also been measured from the dorsal hippocampus to assess correlations with electroencephalographic activity.[61]
The autofluorescence measured by spectroscopic methods is now believed to be dependent on the mitochondrial NADH fraction contained within the volume probed 12, 22, 34 and 50 for the following reasons: (i) NADH absorbs only in its reduced form at 340 nm and fluoresces within the range of blue light at 440–530 nm;[12] (ii) in the gray matter[78] or in the cortex,[34] increased levels of the 450–460 nm autofluorescence are well correlated with increased tissue levels of NADH and the decreased tissue contents in glucose and ATP;[56] (iii) results reported by imaging studies in the surface of the brain also indicate that the blue fluorescence is correlated with the distribution of the mitochondrial NADH fluorescence;[22] (iv) finally, injection of cyanide (cytochrome oxidase inhibitor) increases blue fluorescence while the injection of FCCP (carbonylcyanide-p-trifluoromethoxy phenylhydrazone), which uncouples phosphorylation and oxidative processes, decreases it.[22]
It must also be emphasized that changes occurring in “NADH” fluorescence are complicated by the fact that sampling performed with optic fibre sensors involves the entire tissue sample. Indeed, assignment of the compartment from which the signal arises still remains to be precisely determined, and this aspect has given rise to controversies.19, 22 and 50
Another essential issue concerns the conditions of the animals in reported experimental protocols. It is, indeed, suggested[19] that the “prestimulatory” steady redox state is of paramount importance in determining the direction of NAD/NADH redox balance evoked by activation of the brain cortex. Whether the animals are habituated or not to experimental conditions may lead to decreased or increased in brain contents of lactate and pyruvate.[62] It is also likely that data obtained with anaesthetized animals may not exactly reflect the genuine changes occurring in true physiological conditions.

### 1.2. Sleep aspect

A great variety of data obtained a few decades ago[35] or more recently[10] still point to the leading role played by serotonin (5-HT) in sleep. This amine is synthesized within serotoninergic neurons whose perikarya are located in the raphe system and contribute, together with hypnogenic substances, to sleep preparation, triggering and maintenance.20 and 21 In addition to the aspects relative to neurotransmission or neuromodulation associated with 5-HT or hypnogenic substances, the energetic mechanisms involved in this critical area throughout the sleep-wake cycle and particularly during paradoxical sleep (PS), remain poorly documented. The brevity of the PS episodes displayed by most experimental animals could explain this issue.

### 1.3. Energetic aspect

Energy is generally trapped as ATP by two processes, glycolysis and mitochondrial oxidative phosphorylation, whose respective efficiencies are 6% and 94%.[23] In the rat, glucose is the basic substrate for energy supply and, according to the oxidative phosphorylation processes, is first metabolized within the glial cells into pyruvate which is then transported into neurons.1 and 67 GLUT3 (glucose transporter, isoform 3) is believed to be the major neuronal glucose transporter.[28] At this level, mainly through the pyruvate dehydrogenase complex (PDH), pyruvate is the source for the production of acetyl coenzyme A (AcCoA) and NADH:

$$\mathrm{Pyruvate} + \mathrm{CoASH} + \mathrm{NAD}^+ \xrightarrow{\mathrm{PDH}} \mathrm{AcCoA} + \mathrm{NADH} + \mathrm{H}^+ + \mathrm{CO_2}$$

Through allosteric regulations, PDH is inhibited by the end products and activated by the substrates, i.e. oxygen, Ca2+, pyruvate...[57] AcCoA is the basic fuel for the tricarboxylic acid cycle which produces NADH. Together with oxygen, and through chemiosmotically-coupled oxidative phosphorylation, it is highly ATP productive.[23] It contributes to the synthesis of various chemical species like the fatty acids, the ketonic compounds, acetylcholine (ACh)....[29] NADH is reoxidized into NAD+ through redox processes of the proton motive respiratory chain.6 and 53 It is thus evident that the above aspects of oxidative metabolism depend on, and are reflected by, the redox potential of the cells[31] and that NADH may reflect the status of this aspect.[3]

### 1.4. Sleep and energetic aspects

As yet, the brain energetic mechanisms involved in sleep have not been extensively investigated. It is nevertheless reported that during slow-wave sleep (SWS), glucose [49] and oxygen consumption45 and 49 decrease while glycogen content increases,[37] thus contributing to the restoration of the energy pool.30 and 37 Indeed, SWS is generally assumed to be a restorative state5 and 38 necessary for the occurrence of PS [77] which needs energy to occur. In this respect, on the basis of deoxyglucose (614C) use through autoradiographic technique or positron emission tomography, increases in glucose consumption have been reported during PS as compared to the waking state (W).25, 44, 48 and 60

### 1.5. General purpose

Our experiments were conducted with animals under strictly controlled physiological conditions. Despite the evidence stated above, we first reconsidered the NADH dependence of the in vivo 460 nm signal since this aspect is essential for a sound interpretation of

the data. Afterwards, we examined the relationship existing between the oxidative brain metabolism and the vigilance states. For this purpose, we investigated, throughout the sleep-wake cycle, variations of the 460 nm fluorescence occurring in the PAG-nRD, an area known to contain a large proportion of serotoninergic neurons [79] as well as the PDH complex. 52 and 57 Finally, we also investigated the frontal cortex where the axonal processes arising from the PAG-nRD area impinge. To our knowledge, the time-resolved measurement of brain fluorescence in deep tissue like the PAG-nRD area and its variations throughout the sleep-wake cycle have never been reported.

## 2. Experimental procedures

### 2.1. In vivo experimental procedure

OFA male rats (IFFA CREDO, n=5) weighing 260–300 g were anaesthetized with chloral hydrate (400 mg/kg, i.p.). Two guide cannulas were then implanted in the PAG-nRD and the cortex[58] (Fig. 1). The insertion was performed according to two different angulations (cortex: 45°/frontal plane; nRD: 30°/sagittal plane). The animals were also equipped with electrodes necessary for polygraphic recordings (electroencephalogram (EEG) and electromyogram (EMG): recording of the electrical activity of the cortex and of the neck muscles, respectively). Surgery, electrode production and placement were performed as already described.[20]

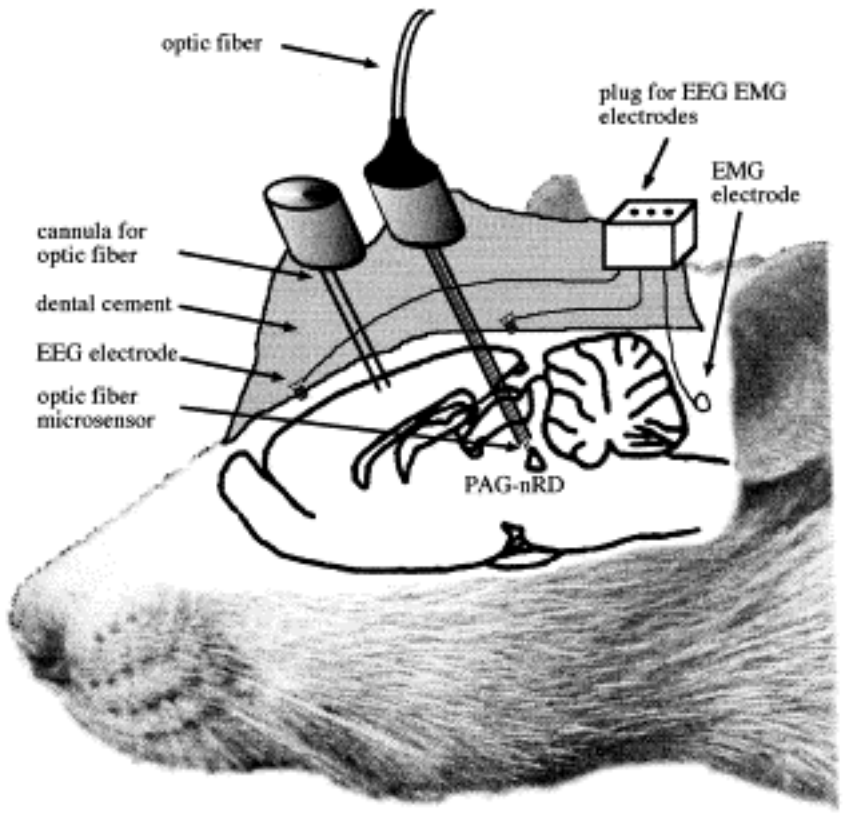



Fig. 1.
Schematic diagram of the optic fibre and the polygraphic electrodes assembly. After surgery, 10 days were necessary for recovery. Constant care was devoted to the animals, the cannula guide was also removed and replaced every day.

After ten days of recovery (12 h:12 h light:dark, temperature at 24±0.5°C, food and water *ad libitum*), the FOCS was placed in the cannula from which it emerged by 2 mm (inner edge). Time-resolved fluorescence measurements, together with polygraphic recordings, started immediately and daily sessions (about 6 h) were possible for more than one month. At the end of each experimental session, the optical fibre was removed from the cannula and washed with milliQ water. *In vitro*, a 15 *μ*M NADH (Sigma) solution at pH 7 (phosphate buffer, Merck) was used as reference before and after *in vivo* sessions. To avoid cerebrospinal fluid overflow between each experimental session, a metallic guide was inserted into the cannula from which it emerged by 2 mm.

### 2.2. Recording sites location

At the end of the different experimental sessions, the animals were killed with a lethal dose of barbiturates (Nembutal), recordings being continued during this step. After death, the position of the working sensor was checked in every animal by applying an anodic current (1 mA/2 s) through an iron electrode inserted into the cannula and also emerging from the inner edge by about 2 mm. Afterwards, the brain was removed, coronal sections (depth: 20 *μ*m) performed with a cryostat and classical Cresyl Violet staining of brain cuts applied.

### 2.3. Data scoring and statistics

Polygraphic data were scored visually as previously described[20] while the fluorescent signals measured were stored in a microcomputer and analysed by means of a home-made program. For statistics, an ANOVA followed by a multiple range test (Bonferroni–Dunnet) were used (Fig. 5).

### 2.4. Time-resolved fluorescence measurements

The experimental set-up used (Fig. 2) has been described elsewhere.[54] Here, we recall briefly that the laser source used was a nitrogen laser delivering pulses of 300 ps (FWHM). The R3810 photomultiplier type (Hamamatsu) was selected in gain in order to detect single events. The 50 Ω output of the photomultiplier was coupled to a transient digitizer (Tektronix Model 7912 AD mainframe, Model 7A19 amplifier unit, Model 7B90P time base unit). The fluorescence signal was averaged during 6 s with a laser repetition rate at 15 Hz. The fibre used was a step index multimode, model PCS 200 (Quartz et Silice, φ core=200 μm). The measurements were volumetric since performed either in the extracellular space or in the intracellular compartment. According to data already published [12] and established by means of an excitation wavelength at 337 nm applied on brain slices, it appeared that the maximal volume probed by our sensor (diameter 200 μm, excitation at 337 nm) was about 0.063 μl. This contrasts with electrochemical methods detecting current at the active surface of the sensor and permeation methods using a membrane as active surface.

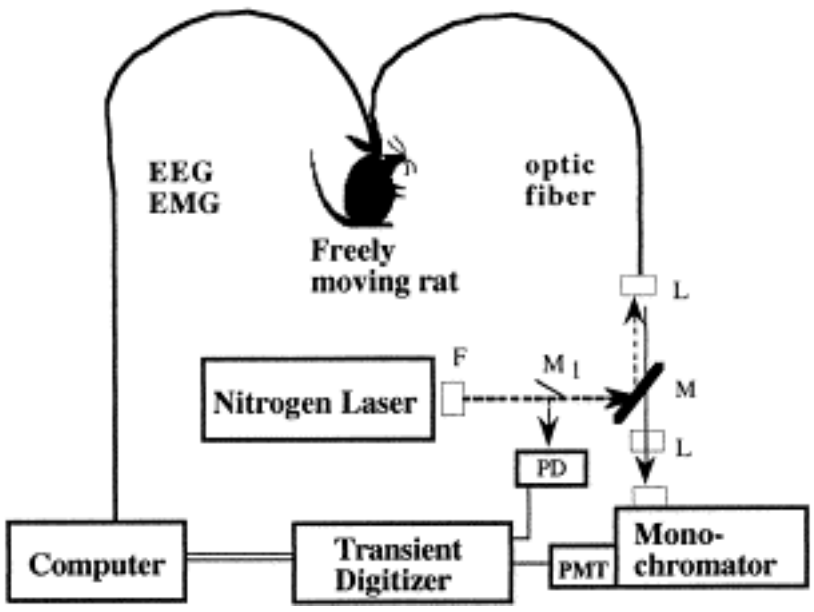



Fig. 2.
General configuration of the experimental set-up displayed for the experiments performed with unanaesthetized and freely moving rats. The experimental sessions were scheduled during the light period (12 h). F, filter; M, dichroic mirror; $M_1$ mirror; L, lens; PMT, photomultiplier; PD, photodiode.

## 3. Results

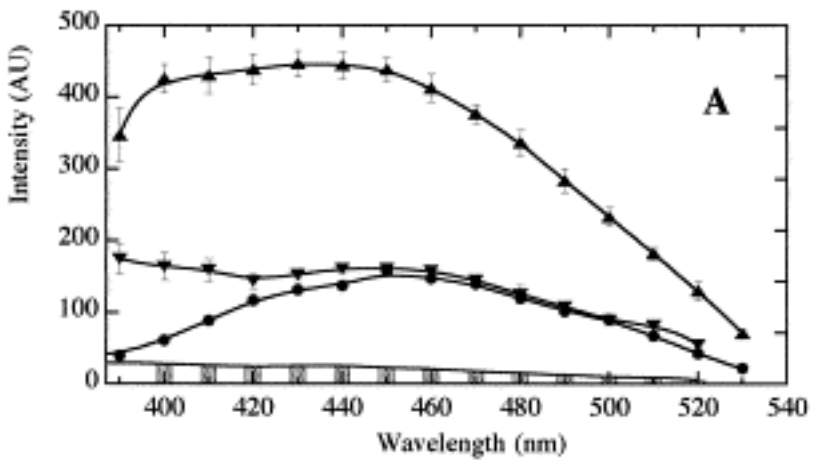
### 3.1. Characteristics of the *in vitro* and *in vivo* signals.

#### 3.1.1. In vitro

A 15 $\mu$M NADH solution (pH 7, temperature=24°C) yielded a typical fluorescence at 460 nm (Fig. 3A). With the oxidized form of NADH ($NAD^+$) no fluorescent emission was obtained.

#### 3.1.2. In vivo

The signals derived from the cortex and the PAG-nRD area appeared in the spectral range peaking around 430–450 nm (Fig. 3A). Their decay times were longer than in vitro ( Fig. 3). Moreover, the fluorescence level derived from the PAG-nRD area was constantly higher than that obtained in the cortex and the magnitude of the ratio PAG-nRD/cortex was 2 (Fig. 3). Finally, in the fluorescence range 390–430 nm, either in the PAG-nRD or in the cortex, an additional signal was present (Fig. 3). According to our in vitro measurements, it might depend on different endogenous fluorophores and a contribution of 5-hydroxyindole compounds is possible. [54]



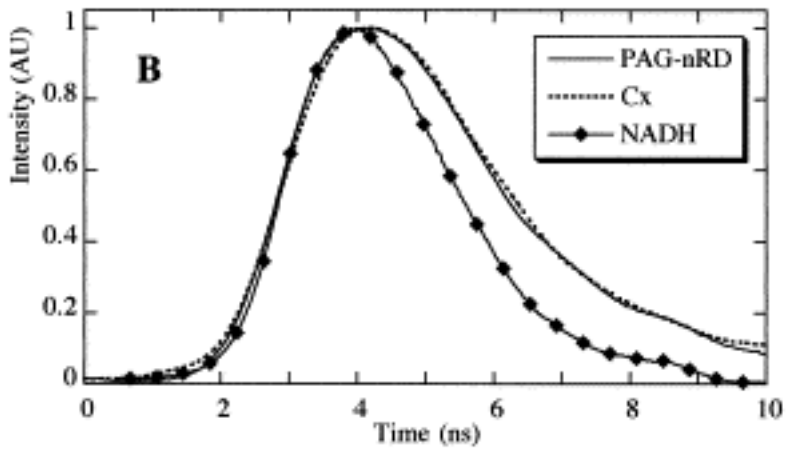




Fig. 3.
A) Typical fluorescence spectra derived through the optic fiber located in; the cortex (P) or the PAG-nRD area (Δ) during a single *in vivo* experimental session; a buffer solution at pH 7 (filled line on grey columns, 1 column=1 experimental point); a NADH solution (O, 15 $\mu$m, pH 7). Bars represent S.E.M. obtained with five measurements. It can be noticed that the signals derived from the cortex and the PAG-nRD area, appear in the spectral range peaking around 430–450 nm while, *in vitro*, the signal derived from a NADH solution appears at about 460 nm; the shift observed *in vivo* is due to the presence of an unknown compound peaking around 400 nm. It also appears that the fluorescence level derived from the nRD is higher than that derived from the cortex. Cx, cortex; NADH, nicotinamide adenine dinucleotide, reduced form). B) Comparison between the typical fluorescence pulses obtained at 460 nm from the two areas investigated (Cx and PAG-nRD) and from a NADH solution (15 $\mu$M, pH 7) at 24°C. It can be noticed that decay times measured *in vivo* are longer than *in vitro*. Wavelength is expressed in nanometers (nm), time is expressed in nanoseconds (ns) and intensity in arbitrary units (AU). For other abbreviations see Fig. 1 and Fig. 3A.

### 3.2. Combined polygraphic and time-resolved fluorescence measurements

#### 3.2.1. Effect of the optic fibre brain insertion

Combined measurements were performed in long-term chronic conditions, i.e. about 10 days after surgery. At the beginning, just after the first brain insertion of the FOCS, the signal obtained exhibited an intensity level which decreases exponentially (typical factor of 1.8) during about 90 min. Afterwards, the signal height increased slightly but continuously for 6–7 hours. After three to five insertions, performed during successive sessions, these phenomena lessened, leading to the occurrence of a signal that decreases just after the probe insertion for about 30 min and remained, thereafter, stable as checked on special sessions extending up to 24 h. The magnitude of the “insertion effect” depends on the probe location and the mechanical stimulation that is inevitably applied during the brain insertion. The background noise, attached to the signals measured in chronic conditions, is mainly due to the laser impulse fluctuations. No noticeable changes occurred in the signal when stretching or rolling-up of the optical fibre was produced by the spontaneous movements of the animal. Such a result is, at least partly, due to the fibre selected which is weakly sensitive to torsions and rotations. Moreover, during experimental sessions the animal was on permanent visual control and its home cage was standing on a rotatable plate which was manually moved to compensate torsions. Such manual compensation was rather unfrequent, i.e. typically once per hour. To further limit spontaneous movements, the fibre was also holded by a rubber wire attached to the ceiling of the home cage. Data obtained have been expressed with reference to the waking state (100%, Fig. 4), since the changes in the signal intensity occurring throughout the sleep-wake cycle were relative from one state to another.

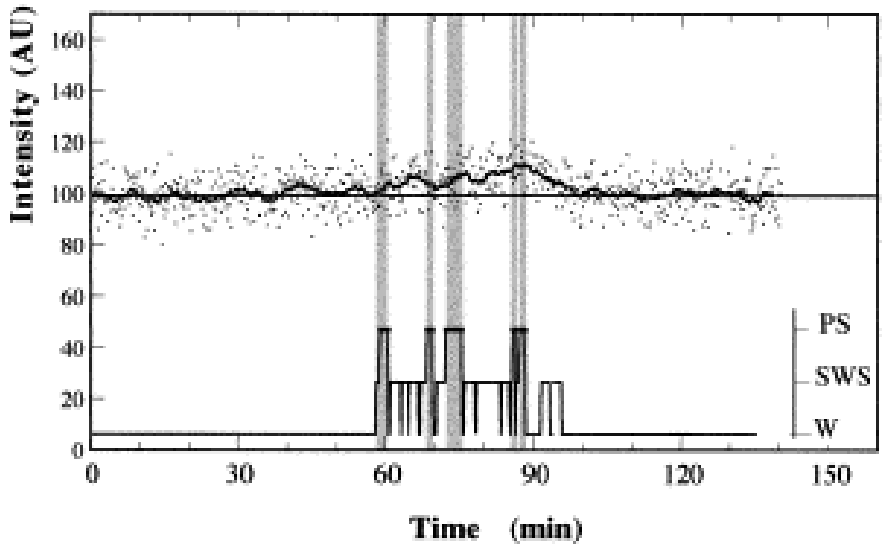



Fig. 4.
Upper part; typical PAG-nRD measurements (fluorescence at 460 nm) obtained throughout the sleep-wake cycle. Data obtained are expressed with reference to the waking state (100%). For each measurement the fluorescence pulses are integrated during 6 s (digitizer full scale at 20 ns). Lower part; hypnogram indicating the vigilance states during which the fluorescence is measured. Note the PS-related increases in fluorescence intensity. The small dots refer to the measured values; the solid line is the best fit. line Abscissae, time in minutes; Ordinates AU, arbitrary units. For other abbreviations see also Fig. 1 and Fig. 3.

#### 3.2.2. Sleep-related changes of the fluorescence in the PAG-nRD

In this area, the intensity of the 460 nm fluorescence varied according to the vigilance state and the location site of the probe (Fig. 5). In its antero-medio-dorsal part (Fig. 5C), a slight decrease was measured during SWS/W (/:versus) while during PS/W a significant increase occurs. In its antero-dorso-lateral part (Fig. 5D), increases in the signal height were observed either during SWS or PS/W. In its ventrolateral part (Fig. 5B), the signal decreased significantly during SWS/W and during PS, while decreasing/W, it increases/SWS. Laterally to the aqueduct, in a site not considered to be part of the PAG-nRD (Fig. 5A), very mild changes occurred during either SWS or PS/W. In this site, the fluorescence level increase was significant during PS/W (Fig. 5A). Finally, outside of the PAG-nRD area and in a mediolateral position, the signal remained stable during W, SWS and PS.

#### 3.2.3. Sleep-related changes of the fluorescence in the cortex

Contrary to what was observed in the PAG-nRD area, in the cortex, the fluorometric signal exhibited homogenous variations, i.e. mild increases during SWS or PS as compared to W. The variation of the signal measured during PS was, however, significantly different as compared to W. Fig. 5.

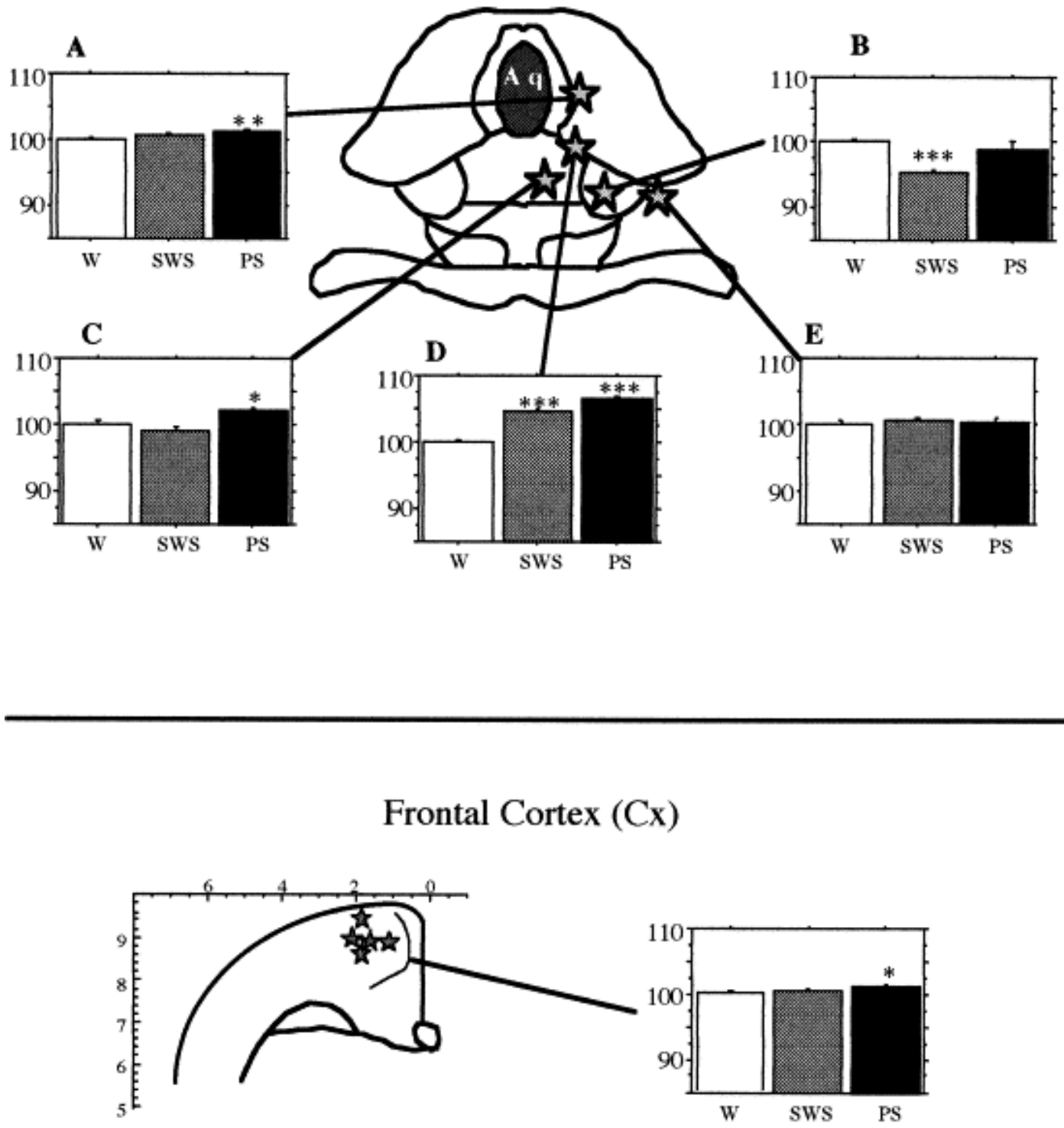



Fig. 5.
Mean variations (±S.E.M.) of the *in vivo* 460 nm signal measured throughout the rat sleep-wake cycle in the PAG-nRD area and in the cortex (1 count=10 s; *n*=number of counts).

| PAG-nRD area | W | SWS | PS |
|---|---|---|---|
| A | 100±0.26 (*n*=599) | 100.5±0.2 (*n*=1 029) | 101.2±0.3 (*n*=402) |
| B | 100±0.35 (*n*=1020) | 99.1±0.47 (*n*=267) | 102±0.59 (*n*=112) |
| C | 100±0.45 (*n*=224) | 99.1±0.47 (*n*=267) | 102±0.59 (*n*=112) |
| D | 100±0.18 (*n*=2 896) | 104.6±0.21 (*n*=1 879) | 106.6±0.46 (*n*=469) |
| E | 100±0.3 (*n*=606) | 100.7±0.26 (*n*=698) | 100.3±0.68 (*n*=101) |
| Cortex (Cx) | 100±0.26 (*n*=600) | 100.5±0.2 (*n*=1029) | 101.2±0.3 (*n*=402) |

For the PAG-nRD, A to E are single recording sites, in each of them measurements were performed during W, SWS and PS. It must be noticed that in the PAG-nRD area the changes occurring in the NADH signal are very heterogeneous. For the cortex (Cx), since the variations measured in each site were very homogeneous, pooled values are reported. W=100±0.26 (*n*=600); SWS=100.5±0.2 (*n*=1 029); PS=101.2±0.3 (*n*=402). It can be underlined that in the Cx, contrary to the PAG-nRD, the changes in the NADH signal are homogeneous. Statistics: an ANOVA followed by a multiple range test (Bonferroni–Dunnet) were used. Comparisons shown in the figure (PAG-nRD and Cx) were performed according to SWS/W and PS/W; significance of the differences is as follows: **P*<0.05; ***P*<0.01; ****P*<0.001; (NS, non-significant differences in E). Comparisons according to PS/SWS (not marked in the figure) are significant in B (*), C (**) and D (***). Aq, aqueduct; see also other figures for abbreviations. Localizations of the sensor (with reference to Paxinos and Watson atlas [58]) are in the frontal plane +1.00 for the PAG-nRD and +9.7 for the Cx. The vigilance state of the animal, determined by polygraphic recordings, is noted at the bottom of each histogram.

### 3.2.4. Variations in the 460 nm fluorescence after a lethal dose of barbiturates

1–2 min after injection of barbiturates, the animal stopped breathing. Shortly after and precisely when the heart beats become irregular, the signal height rose actively for 10 min and then remained stable for another 30 min (Fig. 6). In the cortex and the PAG-nRD, the anoxic/normoxic ratio of the 460 nm fluorescence was in the 1.6–2.4 range. It took about 2.6 min for the signal height increase to occur in the cortex and 4.3 min in the PAG-nRD ($t_m$, fitting function: $1-\exp[-t/t_m]$).

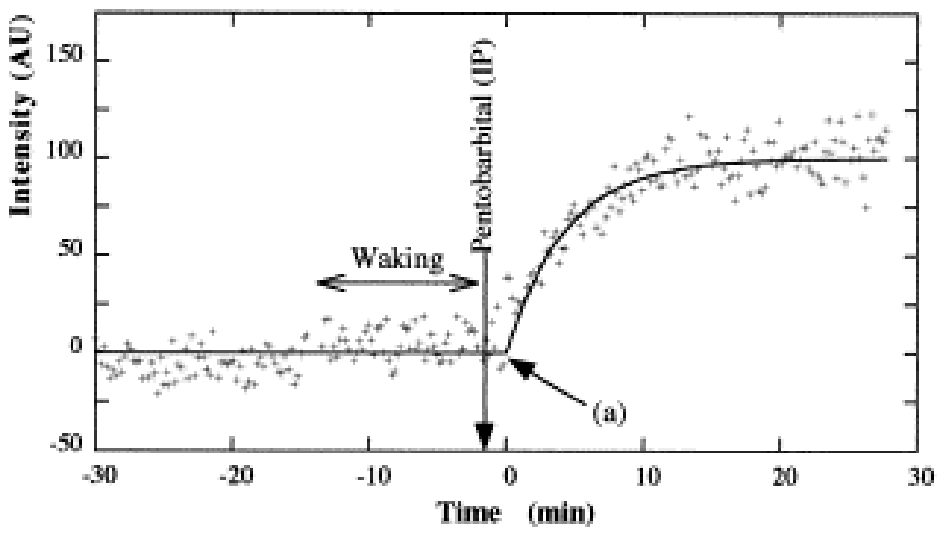



Fig. 6.
Variations in the PAG-nRD 460 nm fluorescence after a lethal dose of barbiturates (i.p.). Mean value of the data was established during the 30 min period preceding the injection (period referenced as 0%) and during the 15–30 min interval immediately following it (period referenced as 100%). The curve fit equation is $y=A\times(1-\exp(-x/\tau)$ and $\tau$=4.31 min in this example. Each dot corresponds to the fluorescence pulses integrated for 6 s (digitizer full scale at 10 ns). (a), breathing cessation; see also other figures for abbreviations.

## 4. Discussion

The present approach has been technically feasible on the basis of three major technical developments: (i) delivery and collection of light signals (laser and fluorescence) through a thin single optic fibre allowing fast measurements (10 s) together with a good anatomical resolution ($\varphi$=200 $\mu$m); (ii) implantation of a guide cannula into specific brain areas allowing the insertion of the optical fibre in the freely moving animal for five to six weeks; (iii) simultaneous data processing of the optic and polygraphic signals derived from the brain.
Particular care was constantly devoted to the animals. Only those in perfect condition were included in the protocol. Moreover, the small diameter of the optic fibre core used enabled us to check the changes occurring in the signal height in limited areas of the brain.

### 4.1. Measurement specificity

The present work reports the changes occurring in the intensity of the 460 nm emission, derived either from the PAG-nRD area or the cortex, throughout the rat sleep-wake cycle. Before discussing the significance of the observed phenomena, it is necessary to carefully analyse the specificity of the measurements performed towards NADH. In this respect, data from the literature, also obtained by means of spectroscopic methods in the brain,12, 32 and 50 are in keeping with the results reported here. Indeed, in vivo, an excitation at 337 nm leads to the appearance of a fluorescent signal analogous to that observed in vitro in the spectral window 460–520 nm with a 15 μM NADH solution. The decay times measured in both conditions appear, however, to be slightly different and this discrepancy might be attached to the fact that, in vivo, NADH exists either in a free form or combined with enzymatic proteins. It is, indeed, reported that the quantum efficiency as well as the mean lifetime of the protein-bound NADH is largely increased as compared to that of the free NADH. 12, 27, 41 and 63 Moreover, the latter form also presents a biexponential lifetime resulting from the complex photophysical properties of nicotinamide and the equilibrium existing at short distance between open/folded conformations of adenine and 1,4-dihydronicotinamide.[16]
However, NAD(P)H, also present in vivo, yields a signal at 460 nm which, at first sight, might contribute to our 460 nm signal. It is, nevertheless, very unlikely since: (i) NAD(P)H-NAD(P) is present in much lower concentrations in the tissue than NADH-NAD+;[45] (ii) the fluorescence derived from the cortex is well correlated with the NADH endogenous concentrations,[34] and (iii) the localized mitochondrial blue autofluorescence is altered by specific inhibitors of the oxidative metabolism.[22] Finally, a perfect control, allowing one to check the strict dependence of the 460 nm signal on the NADH tissue content, would consist in inhibiting its synthesis pharmacologically. Such a procedure, currently used by bioelectrochemists to identify the chemical species in vivo[9] is unfortunately not possible with NADH since the substances altering its production would immediately be lethal for the animals.
Whatever the difficulties, data reported here or available in the literature, support the hypothesis that protein-bound NADH might be the compound that mainly contributes to the 460 nm signal measured in vivo.

### 4.2. Compartment probed

In the brain there are 3 main compartments, i.e. vascular, intra- and extracellular. From the indications mentioned in the present report, it seems likely that when the probe is inserted into the brain, the measurements assess a maximum spherical volume of about 0.063 μl and that, in a first instance, all three compartments might be involved. Regarding the vascular compartment, the limited loss in sensitivity due to blood and observed with unperfused/perfused brain slices [12] can rule out, in our physiological conditions, its significant participation in the signal generation through reflectance and fluorescence. It is clear that haemoglobin (oxygenated and deoxygenated) absorbs at 337 nm (excitation) and 460 nm (fluorescence). In this way, the fluorescence intensity is influenced by tissue blood contents. However, it can be emphasized that the vascular bed is twice less dense in the nRD than in the cortex.[18] It must be also noticed that these interferences are strongly amplified when the experimental conditions do not allow self-homeostatic regulation, as in the case with anaesthetized animals. The present approach overcomes the experimental artifacts attached to the animal preparation, since it was performed in the freely moving rat which self-regulates homeostatic aspects.
Concerning intra- and extracellular compartments, data obtained with cerebrospinal fluid (CSF) indicate that this fluid does not yield a noticeable 460 nm fluorescence signal and is thus devoid of detectable amounts of extracellular NADH.[55] It cannot be excluded, however, that CSF from brain cavities might differ from that contained in the tissue. Anatomical and biochemical data, nevertheless, lay stress on the fact that NADH is an intracellular compound present mainly within mitochondria.12, 22, 34 and 71
Whether the autofluorescence is an index of the free NADH and/or protein-bound NADH is another issue. It is well-known that NADH fluorescence is greatly enhanced by protein binding.27 and 41 A change in autofluorescence measurement can be interpreted as a change in the redox state of NADH or a change in its molecular environment (protein binding, membrane effects...). With our subnanosecond time-resolved fluorescence sensor, we did not observe significant differences in the subnanosecond time shapes of the NADH fluorescence derived either during SWS or PS. It is also reported that cytosolic PDH might be active in ACh

synthesis[75] which is involved in PS executive mechanisms[76] and also released during this state of sleep.[39] Such processes take place during PS and are important for its occurrence. Their contribution to the redox potential is limited since for one AcCoA contributing to ACh synthesis, 200 more are used in the tricarboxylic acid cycle.[75] It appears thus likely that the brain measurements reported here may reflect changes occurring in the redox state of the mitochondrial protein-bound NADH.

### 4.3. Sleep data interpretation

Measurements carried out inside the PAG-nRD area under chronic conditions and reported here, indicate that variations of the 460 nm signal, occurring throughout the sleep-wake cycle, are heterogeneous and dependent on the probe position and the vigilance state. During SWS, according to the site investigated, the signal height either increased significantly or remained stable or exhibited a decreasing tendency. During PS, the changes observed were more homogeneous and significant increases were constantly observed. In the cortex, the signal showed a constant tendency to increase during SWS and PS as compared to W. The fluorescence levels measured in this structure are, however, significant only during PS/W. The variations in the redox potential appear, at first sight, difficult to interpret owing to the myriad of metabolic and transport processes regulating the redox equivalent delivery to the mitochondria. Considerable heterogeneity in the literature data 3, 19 and 71 renders these mechanisms even more difficult to interpret. In contrast with the in vitro preparations and in vivo anaesthetized animals, our results were obtained with animals in perfect physiological conditions in which all the parameters influencing the NAD+/NADH balance were self-regulated by the animal itself, i.e. oxygen supply, temperature regulation, food given ad libitum for the availability of basic energetic substrates. Furthermore, brain NADH is closely linked with ATP production in the rat, mainly from glucose and through the chain of electron transfers (oxidative phosphorylation) in which oxygen is determinant [68]. The balance of the redox potential depends on the oxidative phosphorylation which favours the consumption of NADH, and the PDH and the tricarboxycylic acid cycles which both produce or regenerate NADH. 3, 13 and 23 This aspect is clearly illustrated by the use of a lethal dose of barbiturates which, in reducing the oxygen availability, limits the chemical processes at the AcCoA step and produces a considerable increase in the 460 nm NADH signal. During PS, generally the NADH signal also increases in both areas investigated, i.e. the PAG-nRD and cortex, and the oxygen availability might again be the condition determinant for such an effect. Several reports, indeed, add strength to such an hypothesis since hypoxia decreases PS. 2 and 42 The increase observed in the signal intensity during this state of sleep thus reflects a higher activity of PDH and tricarboxcylic acid cycles directed towards an enhanced production of the oxidative energy. Indeed, by this time, glucose and oxygen consumption increase,[30] consumption of glycogen decreases[36] while lactate concentrations remain unchanged.[69] Thus, in our physiological conditions and particularly during PS, an increase in the NADH 460 nm signal would reflect an activation of the phosphorylative pathway in which metabolic processes from glucose to ATP might be limited by the oxygen availability. This view is again consistently strengthened by the following three sets of data: whether in the rat or in the cat there exists a rise in brain temperature during PS, accompanied by a fall in cerebral blood flow at the beginning of the PS episode, 17 and 64 it is likely that this fall might limit the oxygen availability; chloramphenicol, known to strongly inhibit the first site of the oxidative phosphorylation,[26] very efficiently reduces PS in the cat[59] and in the rat;[14] glucose metabolism is clearly increased during PS.[44] Concerning the latter aspect it can be emphasized that, whether in the cortex or in the PAG-nRD area, the magnitude of the index related to glucose utilization during PS as compared to W[44] is in good agreement with the NADH variations reported in the present paper. These variations appear mild at first sight, but this is not surprising since they reflect the genuine variations occurring in a strict natural situation, i.e. alternation from W to PS. Finally, according to the above discussion emphasizing the importance of the oxidative energy for PS occurrence, it is not excluded that its rate of production and/or consumption might also influence its periodicity.

The data obtained during SWS in specific PAG-nRD sites or in the cortex, where the NADH signal respectively decreases or exhibits non-significant changes, indicate that the consumption of oxidative energy is decreased, as currently suggested during this state of sleep.[30] However, the PAG-nRD probe locations in which the signal increases during SWS might, nevertheless, account for the existence of an oxidative energy consumption during this state of sleep. In this respect, a noticeable release of 5-HT has been reported to take place within these sites during SWS.[10] It should also be mentioned that the PAG-nRD area contains other biochemical components which could require energy during SWS.[21]

### 4.4. Metabolism of the PAG-nRD area during paradoxical sleep

The majority of the 5-HT perikarya is located within the raphe nuclei (77.5%).[79] The largest number is contained within the nRD (52% of the raphe nuclei serotoninergic cells). These perikarya also exhibit different morphological particularities46 and 79 within the whole raphe nuclei. Throughout the sleep-wake cycle, it is now well established that the nRD 5-HT neurons exhibit a regular firing rate during W, which decreases during SWS and even more during PS.8, 24 and 51 This pattern of discharge has been referred to as PS-off.[65] Furthermore, moderate and localized cooling (10°C) of the nRD, which also produces a decrease in neuronal discharge, is sleep-inducing.6 and 7 The iontophoretic application of 5-HT within the nRD decreases the discharge rate of 5-HT neurons.[73] During SWS and PS as compared to W, these neurons are subjected to a somatodendritic release of 5-HT which, through an autoinhibitory process, decreases their discharge rate.[10] The presence of vesicles within 5-HT dendrites provides evidence that 5-HT may be released from them.15 and 18 This dendritic release might be triggered by non-5-HT axonal nerve endings impinging on the dendrites.[10] Finally, in the structures where the 5-HT axonal nerve endings impinge (caudate, cortex, basal hypothalamus), the 5-HT release occurs throughout the sleep-wake cycle in an opposite manner, i.e. maximum during W and decreased during SWS and PS.[10] Otherwise, the significant increase in glucose metabolism, reported to occur during PS,[44] is perfectly in line with our results indicating that the 460 nm NADH fluorescence is increased during PS. It should be noticed that, paradoxically, when the serotoninergic neurons are silent, glucose metabolism increases. The energy consumption might thus be necessary for the processes attached to the dendritic release of 5-HT which could occur through depolarizing processes independent of the soma.[43] In this respect, it is well known that about 50% of the brain ATP is consumed by brain Na+, K+-ATPase and that dendrites require elevated Na+, K+-ATPase activity.23 and 33

## 5. Conclusion

The adaptation of our FOCS in freely moving animals is now efficient for the evaluation of the laser stimulated fluorescence. This procedure, providing rapid assessment of the metabolic changes occurring within defined brain regions with a good spatial resolution, is of wide interest for biochemists, behaviourists and sleep physiologists. The evidence allowing one to suggest that the signal measured at 460 nm might be related to NADH has been discussed. This signal varies according to the sleep states and generally increases during PS in the PAG-nRD area. The changes observed may reflect a state related to the oxidative energy.

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