



**UNIVERSITÀ DEGLI STUDI DI VERONA**

**FACOLTA' DI MEDICINA E CHIRURGIA**

*Dipartimento di Scienze Morfologico-Biomediche*

*Sezione di Anatomia e Istologia*

DOTTORATO DI RICERCA IN

**Imaging Multimodale in Biomedicina**

CICLO XXI

TITOLO DELLA TESI DI DOTTORATO

**An integrated system for video and telemetric EEG recording,  
measuring behavioural and physiological parameters, as a tool  
in Safety Pharmacology testing for assessing CNS-side effects**

S.S.D. BIO/16 – ANATOMIA UMANA

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28 Febbraio 2009

*“Esistono uomini sui quali l’irraggiungibile esercita una particolare attrazione. Di solito non sono esperti: le loro ambizioni e fantasie sono abbastanza forti da spazzare via i dubbi che uomini piu’ prudenti potrebbero nutrire. Le loro armi piu’ efficaci sono la determinazione e la fede. Nel migliore dei casi, questi uomini sono considerati degli eccentrici, nel peggiore dei folli.”*

WALT UNSWORTH  
Everest

*“...Non ero troppo sicuro di cosa potessi raggiungere con la sola tenacia e poche altre capacita’, ma mi ponevo obiettivi molto impegnativi e ogni fallimento non faceva che rafforzare la mia determinazione a realizzare almeno uno dei miei grandi sogni...”*

EARL DENMAN  
Alone to Everest

*All the experiments described into this research work were conducted in the Research and Development Centre of GlaxoSmithKline Verona, Italy, in the Laboratories of Safety Pharmacology Group within the Safety Assessment Department. All experiments were carried out in accordance with Italian regulation governing animal welfare and protection and the European Directive 86/609/EEC, and according to internal GlaxoSmithKline Committee on Animal Research & Ethics (CARE) review.*

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## Objectives and Aim

The aim of present research activities is the setting up, assessment and the validation of a new EEG-Video-Telemetry Model in the Rat. In particular, the potentialities of this model to combine and synchronise the acquisition and the analysis of both physiological-electroencephalographic parameters by telemetry and behavioural patterns by video images was investigated, in order to assess brain's activity both in physiological and pathological conditions.

The main focus of the present research is the potential future application/use of this Model in Safety Pharmacology testing for assessing CNS-side effects, in particular the pro-convulsing liability and sleep disorders following new chemical entities (NCEs) administration.

This research had two principal **objectives**:

1. **Relation between seizures and convulsions.** Analyze the relation between seizures and convulsions (EEGs traces versus behavioural observations) in animals (rats mainly) with the scope to understand the power of the EEG-telemetry Model for assessing the pro-convulsive potential risk of New Chemical Entities (NCEs). This model could refine in Tier II CNS testing the conventional models for pro-convulsive potential assessment (i.e., tests of chemical and electrical induction of seizures).
2. **EEGs traces versus behavioural patterns.** Analyze the relation between brain's electrical activity (the amount of electrical power in the different frequency bands of the EEG) and behaviour in animals for assessing specific CNS side effects. This activity could contribute in the CNS Tier II for the assessment of drug-related sleep disruption and impairments on learning, memory and visual and auditory functions following NCEs administration.

# 1. Introduction

The behaviour is considered to form the ultimate integration of nervous system functions at the level of the intact organism (Mello, 1975; Niesink et al 1998), while the physiology gives insight into phenomena that are hard to see by a simple observation.

Studying this organ system of unparalleled complexity (Santiago Ramon y Cajal, 1897) is often the combination of physiology and behaviour that makes research more interesting and valuable and allows a complete and coherent assessment of nervous system functionalities and alterations.

To properly assess Central Nervous System activities/functionalities a system capable to assess simultaneously the behavioural and the electrophysiological activities of the brain should be used by an integrated acquisition and analysis of EEGs physiological traces and the behaviour in freely moving animals.

## ***Electroencephalography***

The electroencephalography is the neurophysiologic measurement of the **electrical activity of the brain** by recording from electrodes placed on the scalp, on the cortex or on deep areas of the brain. The resulting traces are known as **electroencephalogram (EEG)** and represent so-called **brainwaves**. The EEGs are used by neuroscientists and psychiatrists since 1875 to study the function of the brain by recording brainwaves during controlled behaviours in humans and in laboratory animals, mainly to investigate EEG-sleep and EEG-epilepsy patterns.



*The first EEG recording in human, obtained by Hans Berger in 1929*

**EEG limitations:** electrodes are not sensitive enough to pick out individual action potentials of a neuron, the electric unit of signaling in the brain, or whether the resulting electrical activity is releasing inhibitory, excitatory or modulatory neurotransmitters. Instead, the EEG picks up synchronization of neurons (amplitude of about 100  $\mu$ V on the scalp and about 1-2 mV on the surface of the brain).

EEG has limited anatomical specificity. Some anatomical specificity with EEG topography, which uses a large number of electrodes to triangulate the source of the electrical activity (side effects!).

**EEG advantages:** The time resolution is very high (down to sub-millisecond). The brain is thought to work through its electric activity and EEG is the only method to measure it directly. Newer research typically combines EEG or MEG with MRI or PET to get high temporal and spatial resolution.

### ***Video Recording***

The video image is the most innovative method, which allows to capture the behaviour of alive organisms into their environment.

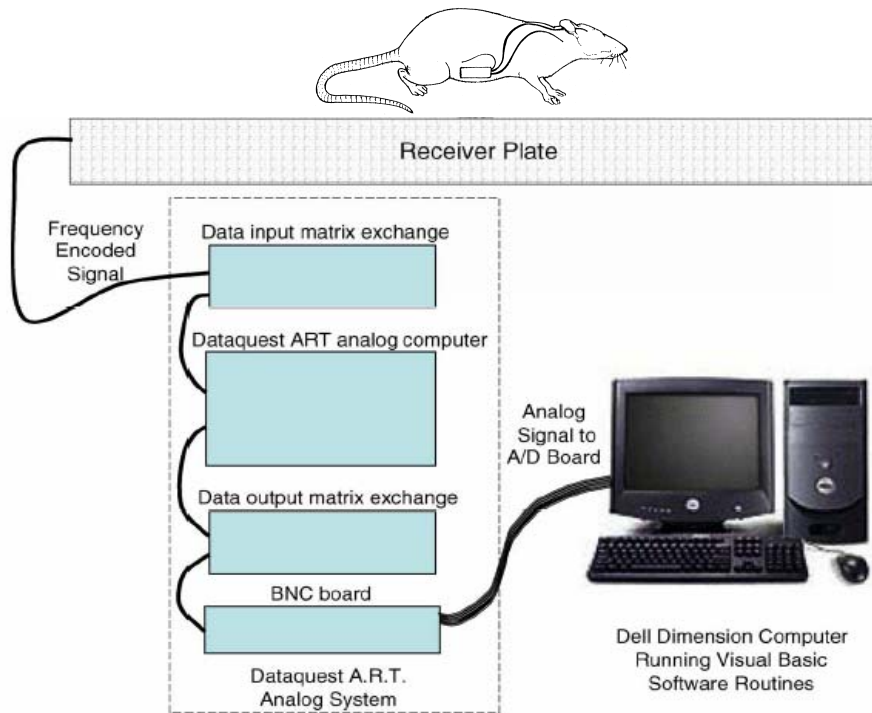
Video recording of behaviour/activity of a freely moving and undisturbed animal can help in the correct definition/analysis and for the improvement of knowledge of behavioural patterns.

The dedicated software-hardware Observer and EthoVision (by Noldus Technology) allow quantitatively and qualitatively measuring and analyzing the behavioural activities displayed by the animal.

### ***Radio-telemetry***

The radio-telemetry is a technology of radio-signals recording from chronically implanted transmitters in freely moving animals widely used to investigate physiological parameters. Telemetry system have progressed over the last 25 years from simple devices allowing the measurement of one physiological parameter to more complex system capable of simultaneously monitoring several variables (BP, ECG or EEG, BT, LA) in large and small animals.

The **fully implantable radio-telemetry** is an alternative technique to that of the non-implantable jacket telemetry and the head tether in obtaining EEG measurements from freely moving animals.



**Figure 1.** A scheme of the telemetric recording system.

### ***Identification of the measuring sites in discrete brain areas***

In general, for the experimental in vivo procedures where the application of electrochemical (i.e., voltametry, micro-dialysis) and electrophysiological (i.e., cell firing, electroencephalography) techniques are used, the [micro]probes are inserted in discrete brain's area by a stereotaxic guide in order to record and analyse the signals which come from this discrete areas.

At the end of the experimental period, it is scientifically relevant and fundamental the correct localisation and identification of the exact position of the surgically implanted active-probe. It is recommended, therefore, to verify post-mortem the anatomical source and the validity of the recorded and analysed signals. The active-probe is the terminal portion or tip of the probe which is involved into the molecular exchange for the micro-dialysis fiber or into the electrical exchange and conduction for the electrodes of voltametry and electrophysiology (metallic probes in platinum, silver, carbonium, stained steel or tungsten) between the tissue and acquisition system. The diameter of the tip of the active-probe can be from approximately 10-20  $\mu\text{m}$  up to 100-200  $\mu\text{m}$ .

The method to identify post-mortem the anatomical position into the deep area of the brain of the active-probe (Jeanblanc J. et al., 2002; Stamford J. et al., 1992; Crespi. F et al., 1988; Borland LM et al., 2005) consists in transferring to the brain's tissue a micro-current



of 10 mA (generated by a generator of current pulses) directly through the deep metallic electrode, which allows the electrical conduction, and applying this current for a short period of approximately 10-15 seconds. This procedure allows generating a slight and local burst of few  $\mu\text{m}$  of neurons around the position site of the electrode's tip. The procedure is performed with the animals in deep anaesthesia.

Then when the animal is still under deep anaesthesia is killed by decapitation and the brain is taken, frozen and preserved at  $-20^{\circ}\text{C}$ . Fresh brains are sectioned with a cryostat in few  $\mu\text{m}$  slices and the Nissl staining method is then used for the detection of Nissl body in the cytoplasm of neurons. The Nissl body of cells is a large granular body found in neurons by the German neurologist Franz Nissl (1860-1919) and is the extranuclear RNA. This method is commonly used for identifying the basic neuronal structure in the brain and spinal cord tissue, because it is able to stain purple-blue the perikaryon in soma and dendrites of neurons, though not in the axon or axon hillock.

## 2. Methodology

### ***The EEG-Video-Telemetry Model***

The EEG-Telemetry Model uses both the Radio-telemetry for physiological parameters recording and video-images technologies. The system allows:

- Making videos and telemetric acquisition simultaneously and synchronised for all filmed subjects;
- Videos and telemetric recording length of 24 hours or more in continuous;
- Start and stop of videos and telemetric data recording can be authomatised (e.g., start during the dark phase without disturb animals);
- Analyze all physiological signals having video images on the screen and the contrary;
- Analyze all videos (ideally automatically) in order to identify specific occurrences/episodes;
- Score manually detailed behaviors around these occurrences synchronized with physiological signals.

A chronically implanted three bio-potential transmitter (TL10M3-F50-EEE by Data Science International DSI) is used for continuous and simultaneous recording of cortical and intracranial/deep EEGs traces, electromyogram (EMG) and locomotor activity (LA) in freely moving adult rats.

This methodological and technical approach allows distinguishing between cortical and limbic (hippocampus) epileptiform activity, considered an essential issue when the initiation and spread of seizures is under evaluation (JF Bastlund et al., 2004, AM White et al., 2006, P Williams et al., 2006). In addition, the use of deep electrodes positioned in specific areas of the brain allows investigations of the electrical activity in relation to specific behaviors in which these areas are known to be mainly involved (e.g., hippocampus for long-term memory processes, amygdale in emotional processes).

## ***Advantages and Disadvantages of the Methodology***

The methodology selected for the EEG-Video-Telemetry Model has the following advantages:

- EEG has a very high time resolution (down to sub-millisecond) and therefore is the only method able to measure directly the very fast brain's activity;
- The quality of the EEG traces is improved by reducing movements artifacts (cable noises);
- Allows prolonged observation periods (continuous sampling) up to 3 months continuously (the duration of the transmitter's battery);
- Avoids methods causing discomfort to animals (e.g., restraint, handling, complications of externalized apparatus, etc);
- Reduces the inter-individual variability; the physiological parameters recorded continuously on the same individual animal also provide chronobiology information;
- Avoids use of other pharmacological entities or electrical stimulation that can cause unwanted effects as secondary lesions resulting from abnormal activity of neurons or acute neurotoxic effects;
- EEG traces directly related to defined aspects of behavior, because animals can be involved in specific behavioral tests and they are completely free to move and respond to different exercises/tests with no differences versus not implanted animals.

And the disadvantages detailed below:

- EEG has limited spatial/anatomical specificity, because electrodes pick up synchronization of neurons (amplitude of about 100  $\mu$ V on the scalp and about 1-2 mV on the surface of the brain);
- More labor intensive, technologically complex and expensive;
- DSI transmitters currently available for small animals generate a maximum of three bio-potential channels, therefore focal seizure initiation and spread can not be investigated (limited anatomical specificity to triangulate the source of the electrical activity);
- EEG-telemetry is very innovative and therefore its successful validation is complex and carries some risks, even if other competitor Pharms seem to be working in this direction in efficacy and safety pre-clinical assessments.

### 3. Experimental activities

#### ***Objectives and Rationale***

The activities described into this research work were associated with the technical and pharmacological validation of the EEG-Video-Telemetry Model, in order to use the model as a tool for the proper assessment of CNS-related unwanted effects.

The model was technically validated in terms of equipment set-up (PART I) and animals' surgical preparation (PART II), as follows:

(PART I) the selected equipment (hardware and software) for the simultaneous and continuous (i.e., periods longer than 24 hours) recording and analysis of physiological parameters (EEG, EMG and LA) by radio-telemetry and the video images by dedicated video cameras was installed and technically verified for their correct functionality.

(PART II) the surgical preparation, the pre- and post-operative care and conditions of animals after the implantation of telemetric devices were tested/refined to improve as much as possible the implantation technique of telemetric devices (model TL10M3-F50-EEE) for physiological signals (e.g., cortical and intracranial/deep EEGs, electromyography EMG) recording and to establish the time required for their complete recovery (in terms of health status and quality of telemetric signals). To this end, the physiological parameters obtained by telemetry and the general activity by videos were recorded continuously and analysed from the day after surgery up to 15 days after surgery (this is the time currently considered necessary for rats to completely recover), in order to follow implanted animals in their post-surgery recovery period.

It was started the pharmacological validation of the model by the preliminary selection of the appropriate doses of known pro-convulsive reference compounds (PART III) and the following use of these compounds on telemetric rats, as follows:

(PART III) Three known pro-convulsing reference compounds (i.e., Pentylentetrazole a GABA<sub>A</sub> receptor antagonist, 4-Amynopiridine a K<sup>+</sup> receptor antagonist and Imipenem/cilastatin a carbapenem antibiotic), were administered to

male CD<sup>TM</sup> rats approximately 12 weeks old, in order to identify the seizure-inducing potentials following systemic administration and to establish the relationship between behavioural and clinical signs preceding and accompanying the convulsive episode and paroxysmal activity of EEG traces.

**Part I:**  
**Equipment Set-up and Technical Verification**

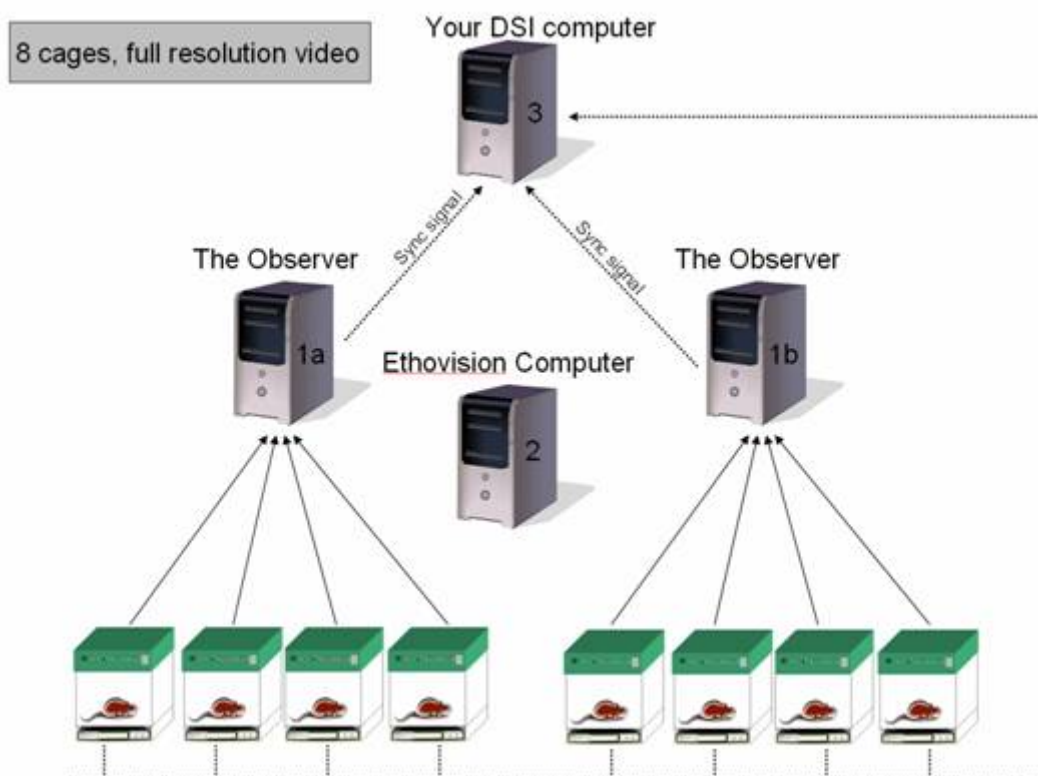
## ***Methods and Materials***

This part of the research was dedicated to select, integrate, install and validate the EEG-Video-Telemetry system equipment.

It has been formed the hypothesis of a system capable to simultaneously assess the animal behaviour and the electrophysiological activities of the brain by an integrated acquisition and analysis of EEGs physiological traces and the behaviour in freely moving animals. This system integrating the two technologies of Radio-telemetry and Video recording should allow:

- Making videos and telemetric acquisition simultaneously and synchronised for all filmed subjects;
- Providing videos and telemetric recording of 24 hours length or more and in continuous;
- Starting and stopping videos and telemetric data recording automatically (e.g., for eventual starts during the dark phase without disturbing animals);
- Analysing both physiological signals and videos having all data on the computer screen;
- Analysing all videos (ideally automatically) in order to identify specific occurrences/episodes of an interest;
- Scoring manually detailed behaviors around these occurrences synchronized with physiological signals.

The initial set-up (Figure 2) proposed by suppliers was refined by a “tests and errors method” to arrive finally at the set up, used to conduct all experiments described in this research work.



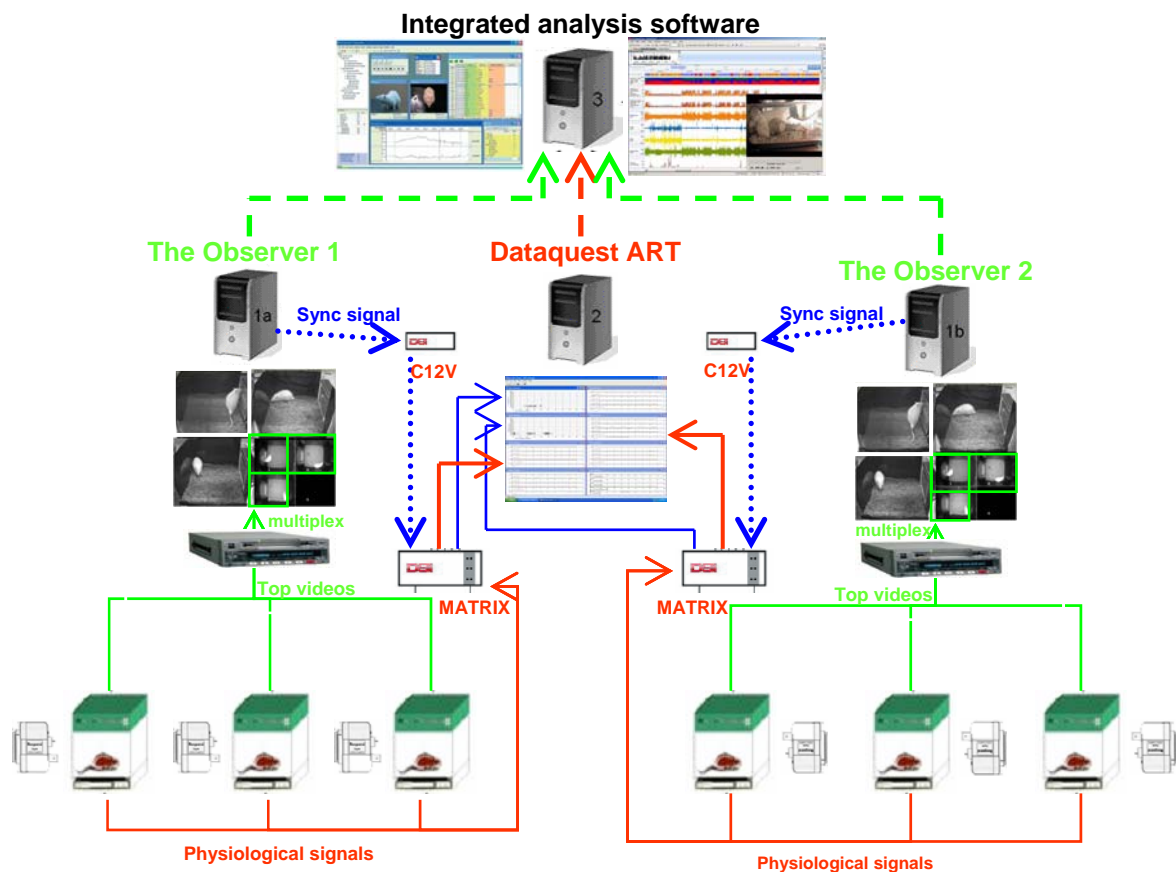
**Figure 2:** The initial set-up for video and telemetric data simultaneous recording.

In the final set-up (Figure 3) the major refinements were:

- the inclusion of an additional camera for each PhenoLab cage (Ikegami black/white camera ICD-49E with Spacecom ottica varifocal manual iris 3-8 mm, F1.4, IR correct) positioned laterally to collect images in full-resolution allowing the detailed analysis of the behaviour;
- the use of multiplexes (Panasonic WJ-MS424 Colour Quad System) for collecting three top-videos in one image, in order to perform on these images the automatic tracking of video data.

The Computer System selected to acquire telemetric signals was the Data Science International *Dataquest ART Gold 4.01*, while the *MPEG4 Encoder* and *The Observer XT* connected directly with *PhenoLab* cages were the selected system to acquire video data.





**Figure 3:** The final set-up for video and telemetric data simultaneous recording.

The components of the installed EEG-Video-Telemetry set-up are:

- Video Acquisition system

- 2 Observer XT with External data module and multiple video module, to control 3 PhenoLab cages (by Noldus Information Technology) each
- 2 The Observer XT multi MPEG-4 Encoder (by Noldus Information Technology)
- 6 PhenoTyper cages for rat with camera top unit (by Noldus Information Technology)
- 1x PhenoTyper Control Unit (by Noldus Information Technology)
- 2 Multiplexers Quad system, each receives images from 3 camera top unit (by Panasonic)
- 6 lateral cameras b/w (by Ikegami)

- Telemetry Acquisition system

- 1 ART Dataquest Gold 4.1 (by Data Science International)
- 2 Interface cable Com port to ART Dataquest (by Data Science International)

- 2 Matrix, each controls 3 receivers and 1 synchroniser C12V (by Data Science International)
- 6 receivers RPM-1 (by Data Science International)
- 2 synchronisers C12V (by Data Science International)

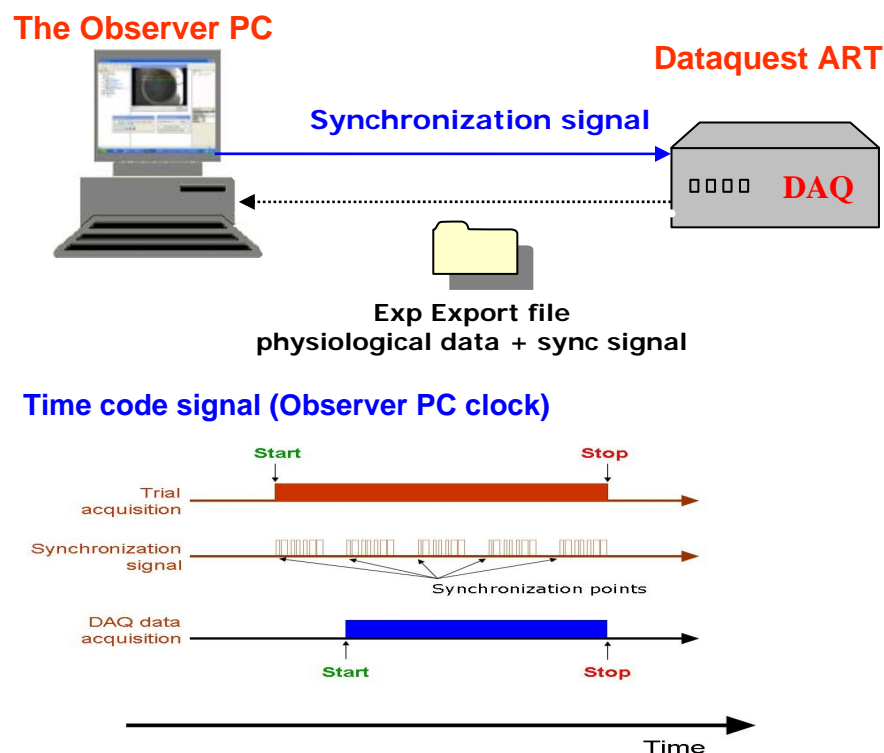
A complete video and telemetric set-up for 6 rats singly housed was installed with videos and telemetric traces recorded simultaneously and continuously for up to 24-48 hrs or longer periods (few days) (Figure 4).



**Figure 4:** The final EEG-video-telemetry system installed in our laboratory.

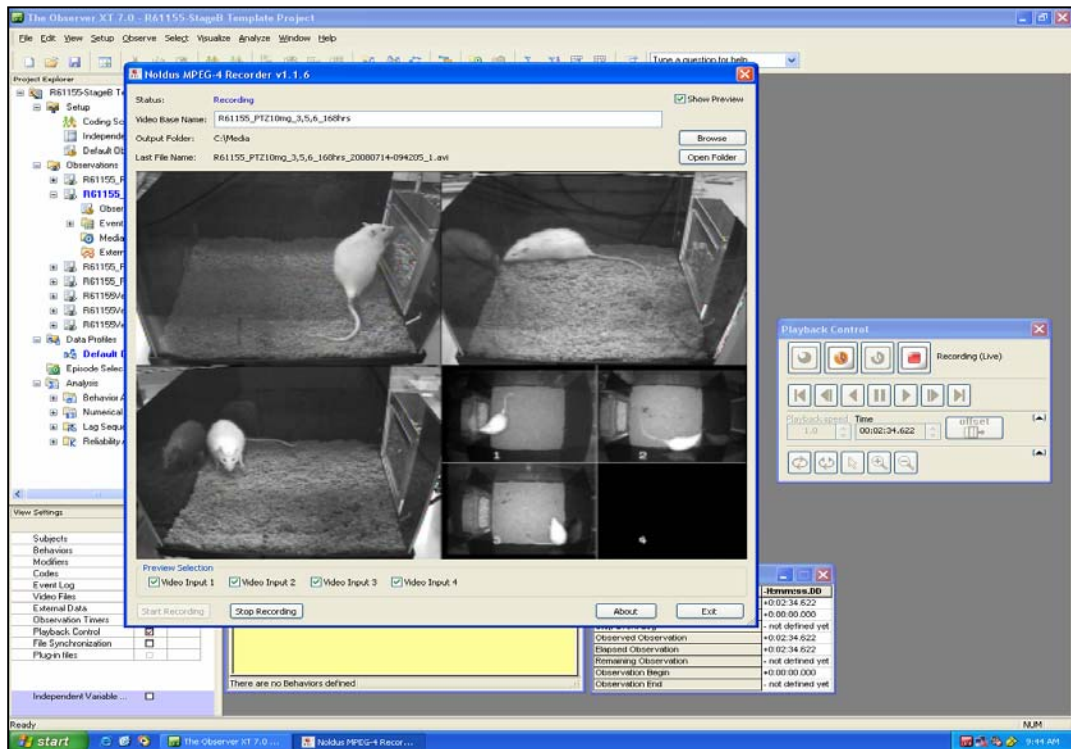
## Data Acquisition and Analysis

The telemetric and video system can acquire synchronised data by a time code signal (corresponding to the Observer PC clock), which is sent continuously during the data acquisition from The Observer PC to the Dataquest ART PC (Figure 5). This time code is then recognised by the Observer software allowing the time synchronisation of the videos with the physiological telemetric data, and all data can be shown on the screen during the analysis.

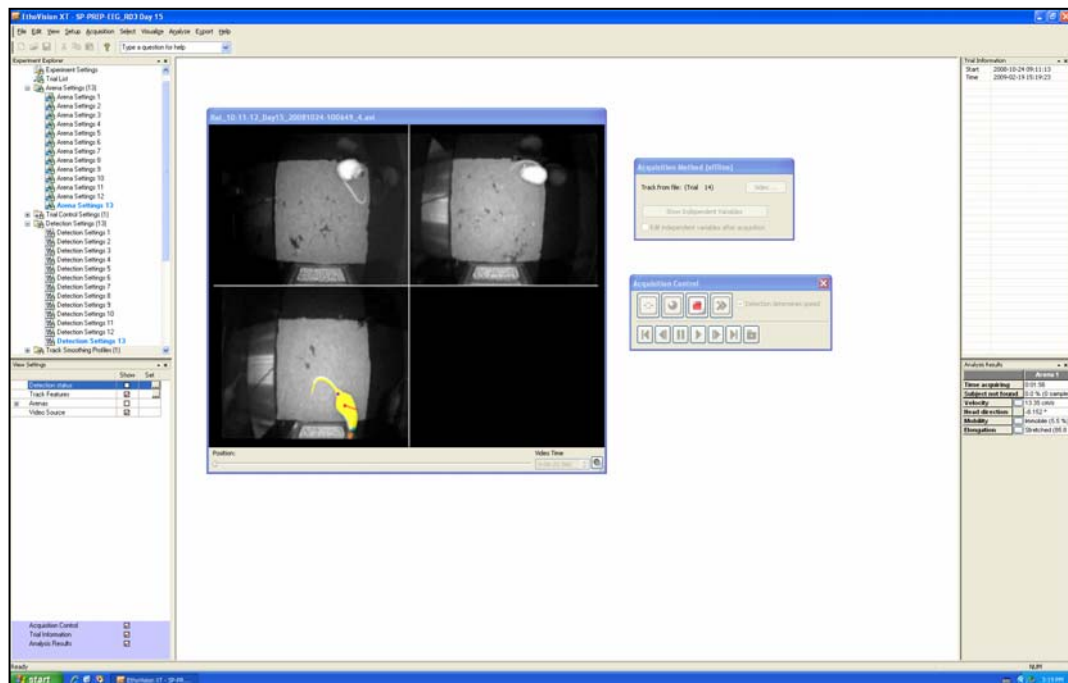


**Figure 5:** How the synchronisation of video and telemetry systems works.

During the acquisition of video data, each Observer system receives video signals from 3 PhenoLab cage cameras top units (3 animals), which are multiplexed to generate a single video signals, and 3 full resolution video signals from 3 lateral cameras (Figure 6). The video images can be first of all analysed with EthoVision XT v5.1 for Windows OS (Noldus Information Technology), which makes an automatic identification of parameters like velocity, distance moved, mobility, movement, elongation thought all the video length (24 hours) (Figure 7). Then, analysis results of the automatic video tracking can be exported into Excel for following statistical analysis and graphs.

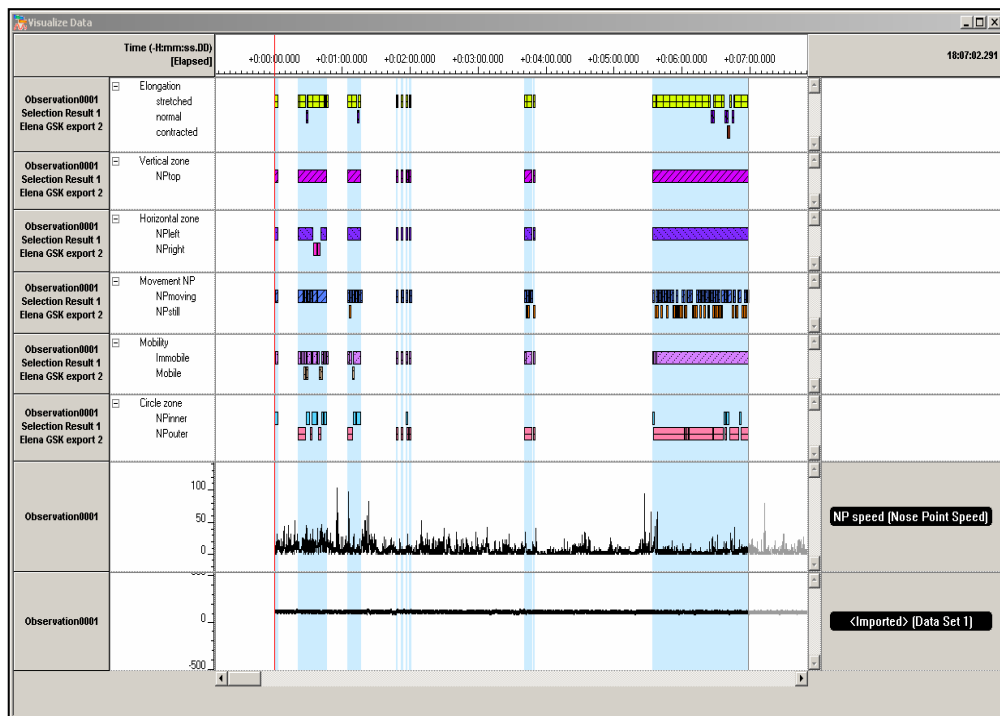


**Figure 6:** The screen of The Observer PC during the acquisition of video signals. The first 3 images come from the lateral cameras, while the fourth image is the multiplexed results of signals from 3 Phenolab cameras top units.



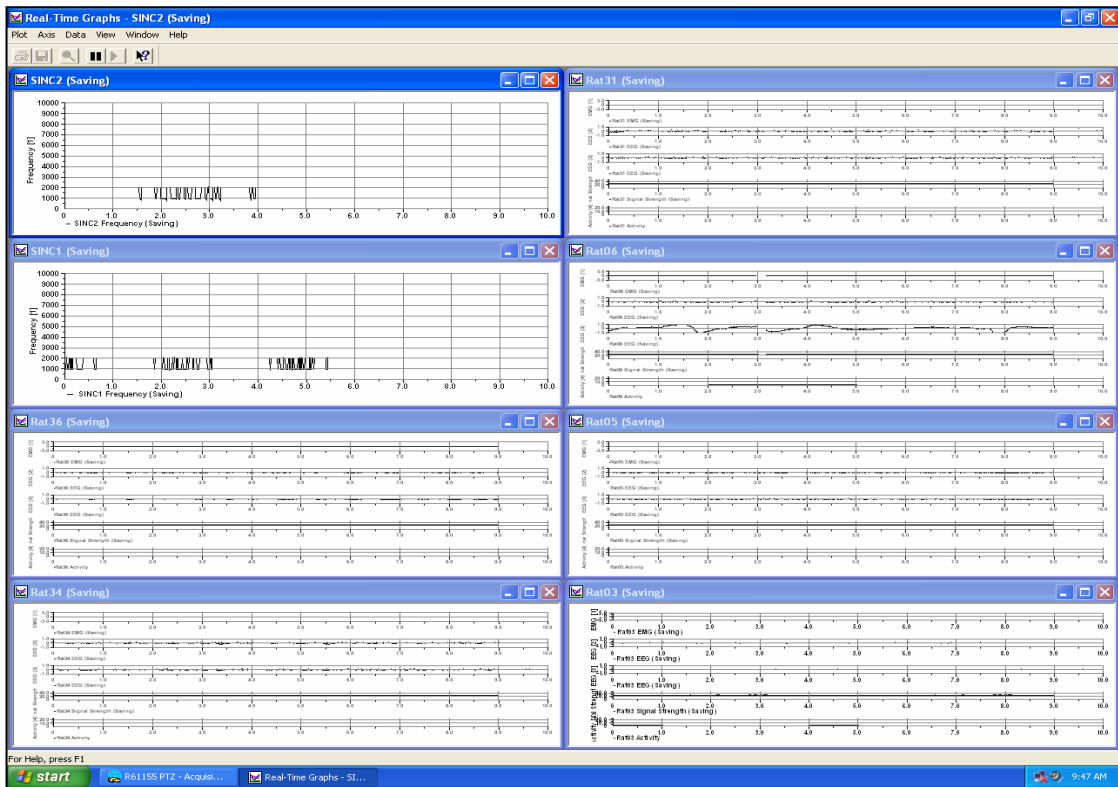
**Figure 7:** The automatic video tracking performed using Ethovision XT software on the multiplexed video signal which come from 3 Phenolab cameras top units.

Video data can also be rearranged for being imported into The Observer XT using an Excel Macro developed by Noldus, which allows identification of specific occurrences/episodes of a particular interest (e.g. episode with high mobility and high velocity, episode with immobility and high elongation, etc). The episodes/occurrences (short video tracts) selected using the Excel Macro or analysing the physiological traces or based on the time, etc, together with synchronized physiological signals can be imported into The Observer XT (Figure 8). Video data can be then analysed manually in order to score detailed behaviors around these occurrences and during this observation-based analysis, having both the images of the animal from the top and lateral cameras.



**Figure 8:** Specific selected episode/occurrences together with synchronized physiological signals (EEGs traces) can be imported into The Observer XT for the manual detailed analysis.

During the acquisition of telemetry data, the ART Dataquest Gold system receives signals from 6 transmitters (6 animals) and the associated receivers which are positioned under the Phenolab cages from 2 synchronisers C12V (quad signals), one for each Observer PC (Figure 9).

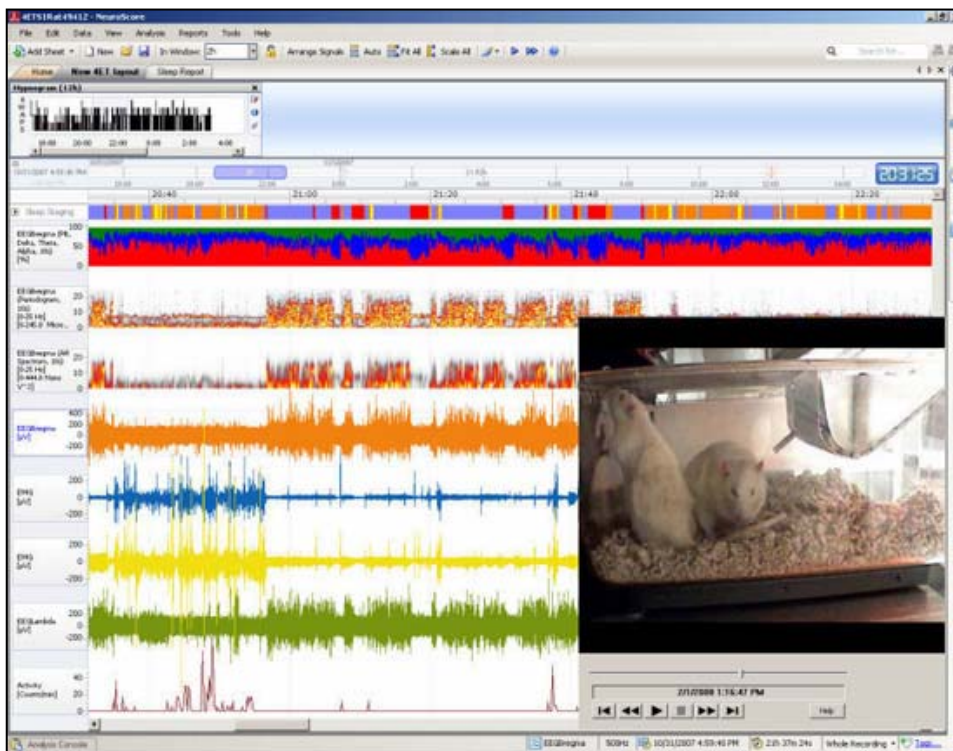


**Figure 9:** The screen of ART Dataquest PC during the acquisition of telemetric signals. The first 2 are the synchronisers quad signals from the 2 Observer PC, the other 6 are the telemetric EEGs, EMG, Activity signals from 6 animals.

The EEGs and EMG physiological traces by telemetry should be ideally analysed having videos on the screen using dedicated software. Video information allows visual assessment of the subject's behaviour, confirmation of scoring events (Weak, Sleep, REM-Sleep), classification of seizure types and more. Currently physiologic waveforms data acquired with Dataquest ART can't be easily imported into an EEG-analysis software (e.g. SleepSign by Kissei America) together with synchronized video data.

Using the Neuroscore DSI software videos and the synchronised physiological traces can be shown at the same time on the screen (Figure 10), but a duplicate video acquisition (in addition to that obtained with The Observer) is necessary, therefore this option was excluded in our current EEG-Video-Telemetry system set-up.





**Figure 10:** The screen of Neuroscore DSI software as appears during the analysis of telemetric signals with the synchronised video data.

## ***Discussion***

The video-telemetric set-up allows to analyse the physiological traces by telemetry using dedicated software (i.e., Sleep-Sign, Neuroscore) with videos on the screen, and also it allows analysing the video images using dedicated software (i.e., Ethovision and The Observer) with telemetric traces on the screen.

The installed EEG-video-telemetry set-up has shown the following advantages:

- EEG traces directly related to defined aspects of behaviour and animals can be involved in specific behavioral tests;
- the quality of the EEG traces is improved by reducing movements artifacts (cable noises);
- prolonged observation periods are allowed (continuous sampling);
- methods causing discomfort to animals are avoided (e.g., restraint, handling, complications of externalised apparatus);
- the inter-individual variability is reduced because physiological parameters and behavioural activities are recorded continuously on the same individual animals also providing chronobiology information.

Also limitations have been evidenced by the system set-up:

- Labor intensive and technological complexity;
- DSI transmitters currently generate a maximum of three bio-potential channels (electrical activity of more than two-three discrete brain's areas can not be investigated in small animals);
- Noldus video recording allows a limited number of videos for each Observation system (a maximum of 3 animals - 3 top multiplexed + 3 lateral videos - or 4 animals - 4 top videos at full resolution);
- Integration of acquired data is not fully complete/free and easy (e.g., not simple and/or possible to import video data acquired with The Observer XT into the Sleep, Epilepsy analysis software together with synchronised DSI physiologic data; DSI data must be converted into .txt files (hours are request!) to be imported into The Observer XT);
- Large amount of video data (storage and archive considerations!);
- The technological complexity of the EEG-video-telemetry system makes hard the GLP-validation.



**PART II:**  
**Animals' Surgical Preparation**

## **Materials and Methods**

### **Animals and Housing**

Twenty-three male CD<sup>TM</sup> rats (CrI:CD (SD) IGS BR) supplied by Charles River Italia, Calco (LC), Italy were used for surgical implantation of telemetric devices. They had approx. 7 to 8 weeks age on the day of surgery and a body weight of approx. 225 to 250 grams.

An acclimatisation period of 5 days before surgery was applied when animals were housed in groups of 4 into solid bottomed plastic cages with sawdust litter. They were housed into solid bottomed plastic cages with clean absorbent tissue (alpha-dri) during the first 8 days of the recovery/post-surgery period and into PhenoTyper cages with clean absorbent tissue (Cellu Dri Soft) during the days of the recording of data.

A temperature range of 20-22°C (19-23°C for less than 24 hours) and a relative humidity range of 45-65% (40-70% for less than 24 hours) with fluorescent lighting illumination from approximately 06:00 to 18:00 hours daily were applied as husbandry environmental conditions.

As diet Gamma irradiated Rat and Mouse No. 1 Expanded Diet (Special Diet Services [SDS] Ltd., England) was provided *ad libitum*. Drinking filtered water from normal domestic supply was provided *ad libitum*. Animals were not fasted prior to surgery and they had given access to food and water approximately 1 hour after surgery.

### **Antibiotic cover and analgesic treatment**

The day of surgical preparation animals received:

- Rimadyl<sup>TM</sup> (Carprofen 50 mg/mL) 5.0 mg/kg (sc) at 1mL/kg, Pfizer Inc. U.S.A.; animals received one subcutaneous dose immediately before surgery;
- Rubrocillina<sup>TM</sup> Veterinaria, Nuova ICC, Italy at 0.1 mL/rat (benzylpenicilline benzatinica 125,000 U.I./mL plus dihydrostreptomycin sulphate 50 mg/mL); animals received one subcutaneous dose immediately before surgery;
- Sodium Chloride 0.9% w/v solution, 5 mL/animal was administered subcutaneously immediately after surgery.

Starting from the day of surgical preparation (Day 1) until Day 3 animals received Rubrocillina<sup>TM</sup> Veterinaria 1.0 mL/kg (sc) and Rimadyl<sup>TM</sup> 5 mg/kg (sc) once daily.

### **Telemetric probes implantation**

Animals were selected for surgery on the basis of their clinical conditions and general behaviour. Implantation was performed under aseptic conditions, anaesthesia was

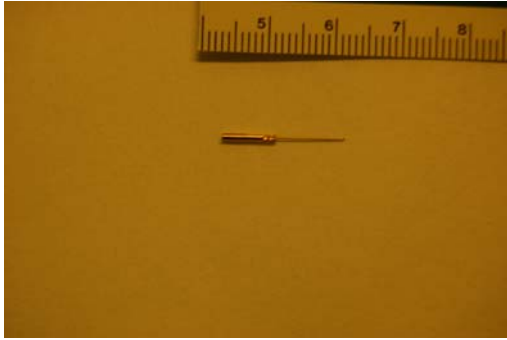
induced by 5% isoflurane (Forane, Abbott) vaporised in 1.5-2 L/min O<sub>2</sub> and was maintained throughout the surgical procedure under 1.5-2% isoflurane anaesthesia (Forane, Abbott), vaporised in 1.5-2 L/min O<sub>2</sub>; this level of isoflurane was adjusted as required to maintain an appropriate level of anaesthesia.

### **Material preparation and general information**

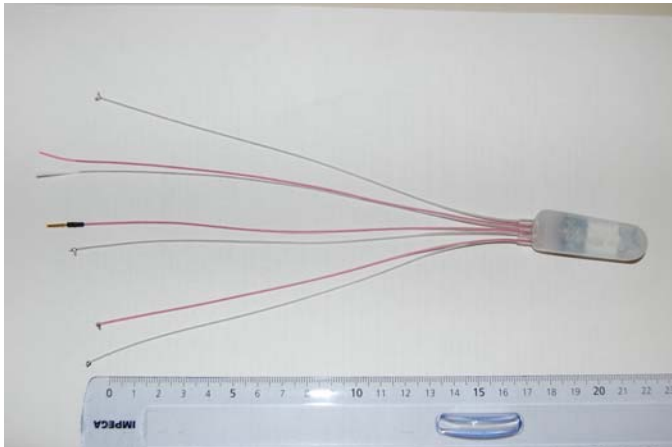
All instruments and accessories were be autoclaved before use. During the surgical procedure the body core temperature of the animal was be maintained with a homoeothermic blanket system, thermostatically controlled (regulated at  $37\pm 1^{\circ}\text{C}$ ) by means of rectal thermocouple. The surfaces over and around each of the incision sites was shaved and the skin treated with Betadine solution (Iodopovidone 7.5 g/100 mL - 10% iodide – glycerine, nonilfenossipoliethilenoxyethanol sodium phosphate, citric acid monohydrate; ASTA Medica S.p.A., Italy) and then with Citrosil Alcolico Azzurro (Benzalkonium chloride 2.5 mg/mL, ethylic alcohol 700 mg/mL; L.Manetti, H. Roberts & C. Italy). The animals were then secured to the operating table to allow access to all incision points and covered with adhesive incise drapes to avoid contact with any non-sterile surface by the operator. The implant was removed from its sterile packaging or from the sterilising solution (2.5% gluteraldehyde solution, overnight) and submerged in sterile saline for at least 15 minutes before implantation.

### **Transducer Preparation**

The three-channel PhysioTel® Multiplus series transmitter type TL10M3-F50-EEE (Transoma Medical, US) was used. For all leads the silicone coating was peeled back to expose approximately 5 mm of the helical steel lead. The tip of the ground electrode, the negative and positive leads of channel 3 (cortical EEG biopotential) and the negative lead (white) of channel 2 (hippocampus EEG biopotential) were yielded to create an angle of approximately  $90^{\circ}$ . A deep electrode (stainless steel electrode E363/2, Plastics One) was crimped onto the positive lead (red) of channel 2 (hippocampus EEG biopotential) (Figures 11-12). Electrodes of channel 1 was used for EMG recording.



**Figure 11.** The stained steel electrode used to record hippocampal EEG.

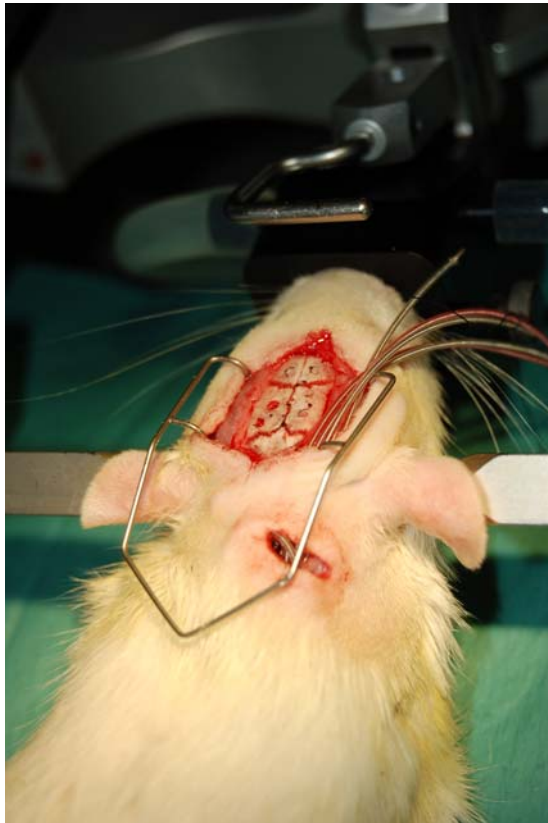


**Figure 12.** The telemetric transducer type TL10M3-F50-EEE prepared for cortical EEG (channel 3), hippocampal EEG (channel 2) and EMG (channel 1) recordings.

### **Surgical Transducer Implantation**

The animal was placed on the heating pad in supine position and a 5-6 cm midline abdominal incision was made through the skin and muscle along the *linea alba*. The transmitter body was placed into the peritoneum parallel to the long axis of the body with the leads directed rostral and it was fixed to the inner peritoneal wall with 4-0 silk suture. The three-couple of leads were passed subcutaneously through the muscle abdominal incision, and then the incision was closed using absorbable suturing thread (Vicryl 3-0).

A 2 cm lateral incision was made just below the shoulder blades, the site of the EMG leads placement. A 2 cm mid-sagittal incision was made on the scalp from the bridge of the nose to the posterior end of the cranium, the site of the two-couples of EEG leads placement (Figure 13). The two incisions were temporary protected/covered using saline (previously heated to approximately 37°C) moistened gauze sponges. A hollow trocar was used to tunnel subcutaneously through the flank region the leads, directed to the incisions on the head and the neck of the animal.



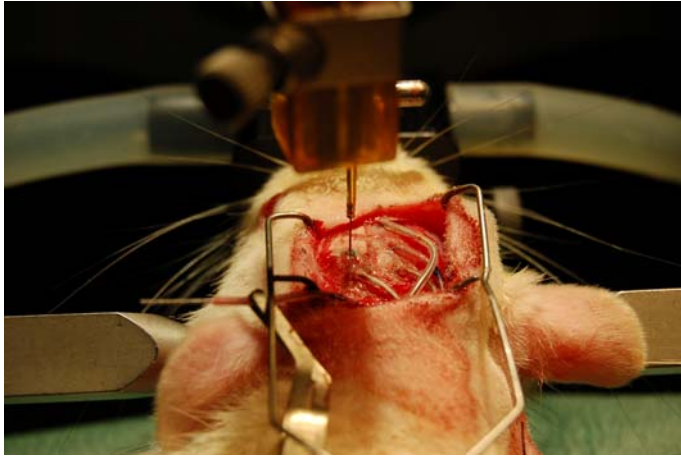
**Figure 13.** Two incisions for placement of the EEGs leads on the scalp and EMG leads on the back of the neck for EMG.

The two electrodes for EMG recording were secured to the underlying *musculus cervicoauricularis* (neck muscle) by 4-0 silk suture. The skin incision was closed using absorbable suturing thread (Vicryl 3-0).

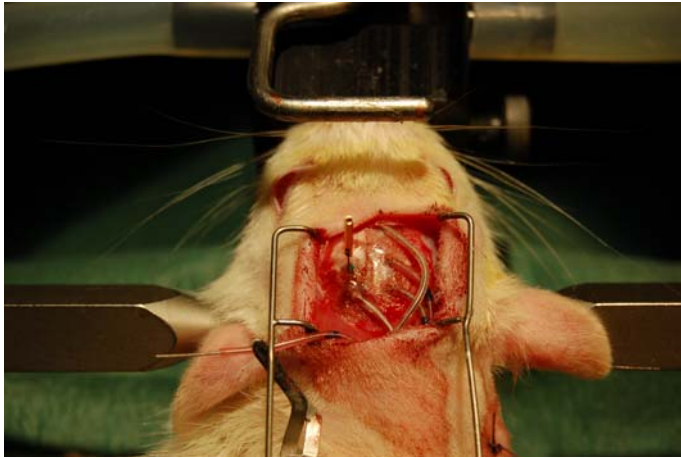
The animal was then secured in the stereotaxic apparatus. The skin on the scalp was reflected using a retractor to expose the entire dorsal portion of the skull. The periosteum was removed and haemostasis achieved with sterile cotton-tip applicators and a cautery pen where necessary. Five holes were drilled in the skull surface. One hole was used to position the ground lead placed supradurally over the right frontal lobes behind Lambda.

The two electrodes for the cortical EEG recording were placed supradurally on the right hemisphere, both 3mm lateral to bregma and the positive lead (red) at 2mm and the negative lead (white) at 6mm posterior to bregma over the parietal association cortex (red: AP -2.0mm, L 3.0mm ; white: -6.0mm, L 3.0mm). The two electrodes tips were pressed into the predrilled holes in the skull, touching the dura, and were initially hold in place with a drop of tissue adhesive (3M Vetbond, St.Paul , MN, US) and a small quantity of gel cyanoacrylate gel (RS Components, Northants, UK).

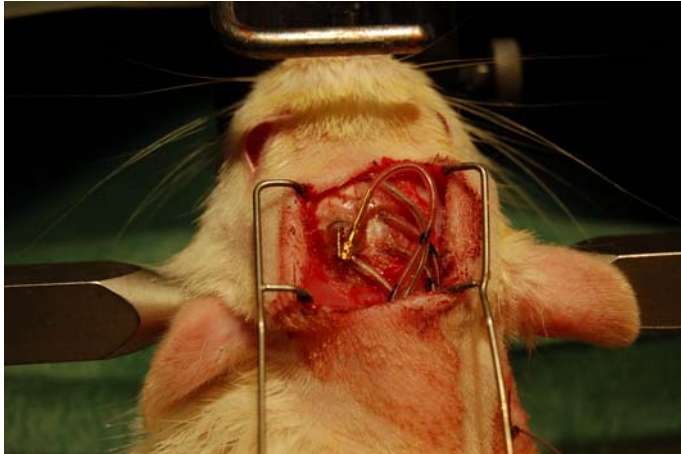
An intra-hippocampal 10mm length stainless steel electrode (E363/2 by Plastics One) was attached to a guide wire and using stereotaxic coordinates (AP -4.0mm, L -2.5mm, V 3.8mm), was placed deep in the granule cell layer of the dorsal dentate gyrus of the left hippocampus and was initially hold in place with a small quantity of cyanoacrylate gel (RS Components, Northants, UK). Then the electrode was connected to the positive radio-telemetry lead (red) by direct mechanical contact. The negative lead (white) was placed supradurally at approximately 3mm lateral and 7mm caudal from the hippocampus lead (AP -7.0mm, L -3mm) (Figures 14-15-16).



**Figure 14.** The intra-hippocampus electrode attached to a guide wire is positioned deep in the left hippocampus.



**Figure 15.** The intra-hippocampus electrode is hold in place with a small quantity of cyanoacrylate gel.

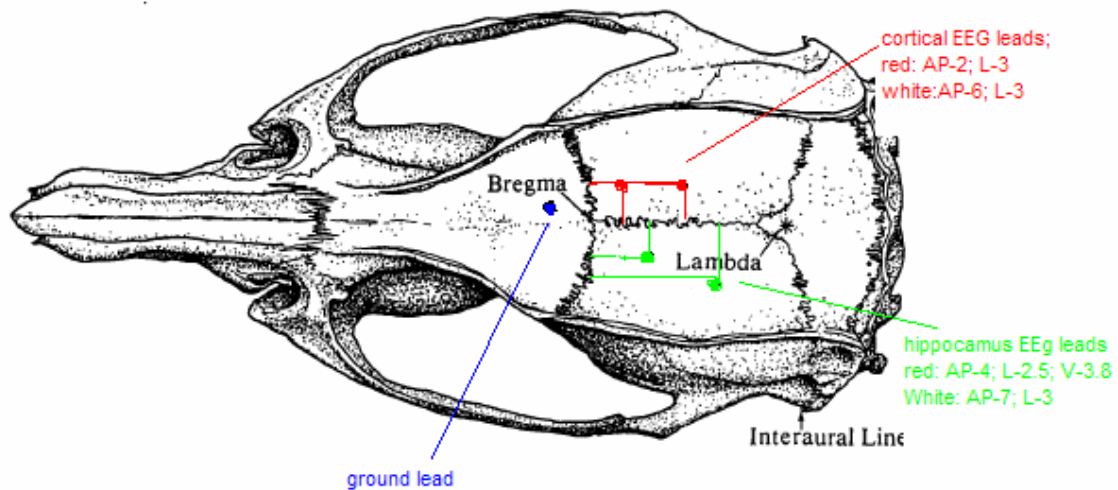


**Figure 16.** The intra-hippocampus electrode is connected to the positive radio-telemetry lead (red) by mechanical contact. The negative lead (white) is placed supradurally caudal from the hippocampus lead.

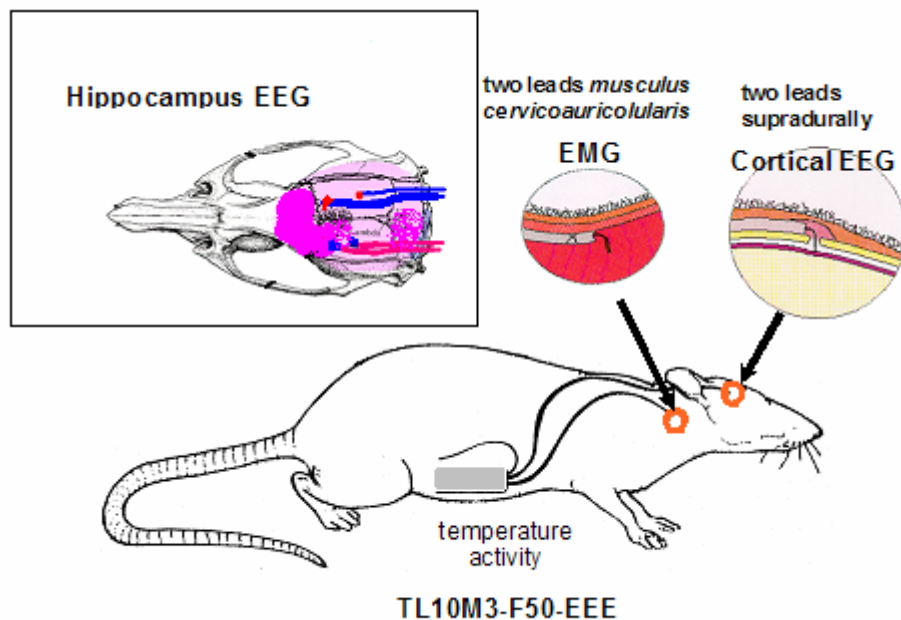


**Figure 17.** All electrodes are covered and fixed to the skull with dental acrylic cement.

All electrodes were then covered and fixed to the skull with dental acrylic cement (Denture Reline Material self-curing by SC Stratford-Cookson Company) (Figure 17). The skin incision on the scalp was closed using absorbable suturing thread (Vicryl 3-0). The excess lead was placed in a pocket under the abdominal skin to prevent tangling of leads and intestines. Finally the skin abdominal incision was closed using absorbable suturing thread (Vicryl 3-0). After completion of the transmitter implantation, no external device is visible (Figure 18-19).



**Figure 18:** A scheme of stereotaxic positioning of all the telemetric leads on the cranium.



**Figure 19:** A scheme of the surgical implantation of the telemetric transmitter in the rat.



### **Post-surgical recovery**

At the end of the surgery the abdominal suture was moistened with Citrosil Alcolico Azzurro (benzalkonium chloride 2.5 mg/mL, ethylic alcohol 700 mg/mL; L.Manetti, H. Roberts & C. Italy). After surgery the animals were placed in the incubators with warm environment and observed until they were awake.

### **Recording and analysis of videos and telemetric signals**

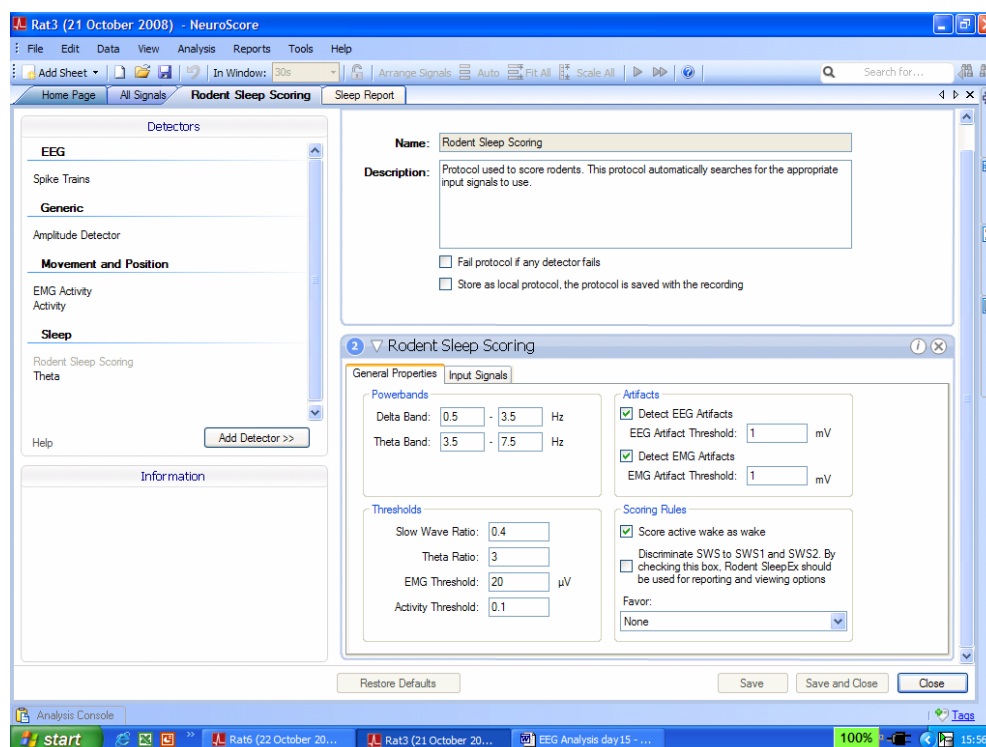
On 23 implanted animals, EMG, cortical EEG and hippocampus EEG signals by telemetry and the general activity data by video were recorded during the recovery/post-surgery 15 days period to assess the quality of the signals and to collect background data on the recovery process from this type of surgery. In particular, recordings of these parameters were made on each occasion continuously for approximately 24 hours on Day 1, 6 and 15 after surgery.

Video data were collected using the *MPEG4 Encoder* and *The Observer XT* connected directly with *the PhenoLab cages* (Noldus Information Technology). Video data were analysed using the automatic video-tracking software *Ethovision XT*. From the 23 subjects, 19 rats were selected and for both the whole 24 hours period and in time bins of 30 minutes of length the following parameters were evaluated and reported:

- Distance moved (the distance of the animal's center-body point movement);
- Elongation (the time spent by animals in a contracted, normal and stretched body position, with thresholds at 80 and 20%);
- Mobility (the duration of mobile animal, with thresholds at 3 - immobile);
- Movement (the duration of moving of the center-body point);
- Velocity (velocity of the animal's center-body point, with thresholds at 2 and 1.7 cm/s).



Telemetric data were collected using the hardware-software acquisition system *Dataquest ART Gold 4.01* (Data Science International). Telemetric EEGs and EMG traces of 7 implanted rats were analysed for the whole 24 hours period and sleep stages wake (W), sleep (SWS) and REM-Sleep or Paradoxical-sleep (P) were evaluated using the software *Neuroscore v.2.0* (Data Science International) and applying the *Rodent Sleep Scoring* detector (Figure 20).

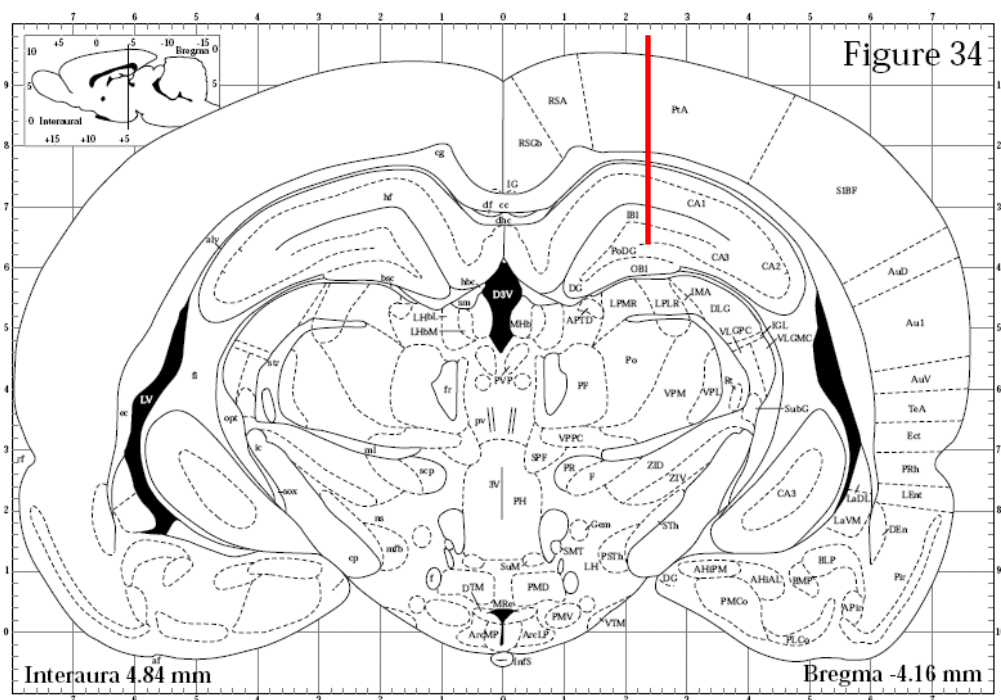


**Figure 20:** The Rodent Sleep Scoring detector applied to telemetric data for sleep analysis.

Using the software *Neuroscore v.2.0* also an automatic identification of electrical artifacts duration on the telemetric traces was performed. In addition a manual-visual verification of the quality of signals was performed to compare telemetric traces recorded on Day 1 and those recorded on Day 15.

## Post-mortem investigation

Under deep isoflurane anaesthesia a low current of 10mA for approximately 10-20 seconds were administered through the deep electrode at all animals involved in previous experimental activates; then the animals were killed by decapitation. The brain was taken, frozen in an isopentane solution (approximately -30 °C) and then preserved at -20°C. After cryostat sectioning of 14µm slices, Nissl (cresyl violet)-stained histological sections were made in order to identify the correct positioning of the deep electrode into the left hippocampus (*dentate gyrus*) at -4.16 mm from the Bregma following the coordinates of the Paxinos & Watson Atlas (Figure 21).



**Figure 21:** The desired position of the deep electrode into the left hippocampus (*dentate gyrus*) based on stereotaxic coordinates of the Paxinos & Watson Atlas.

## Results

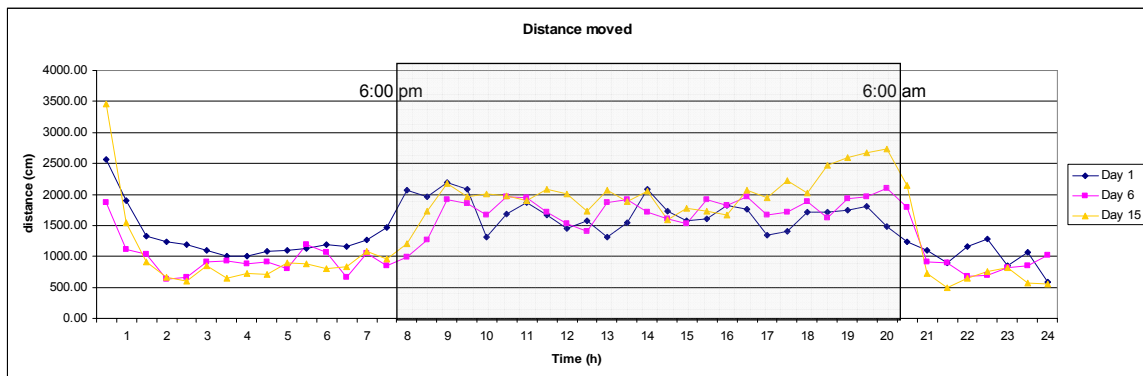
### Analysis of the videos

The analysis of video data during the 24 hours period comparing Day 1, Day 6 and Day 15 showed no significant changes in the mean distance of the animals' movement (Graph 1) during the whole recovery period.



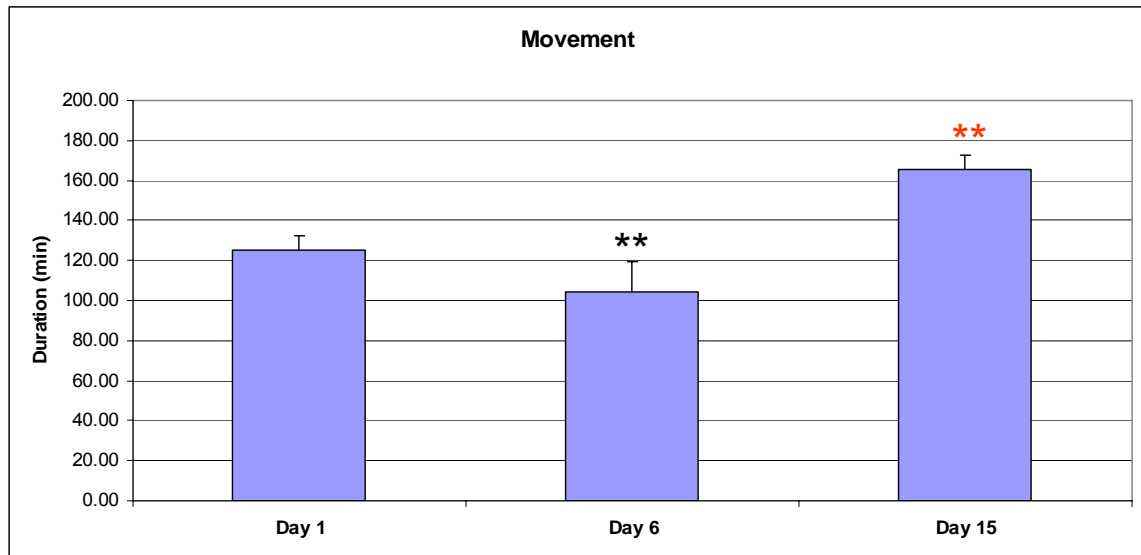
**Graph 1:** Mean distance moved and standard errors during the whole 24 hours period.

From the analysis in time bins of 30 minutes of length was noted a trend to increase the distance of locomotion from Day 1 to Day 15 after surgery and on Day 15 during the last hours of the activity phase (dark phase from 6:00 pm to 6:00 am) the distance of movement was particularly high. However, this parameter showed that the light-dark circadian cycle, with rats less active during the day and active during the night, was maintained starting from Day 1 after surgery (Graph 2).



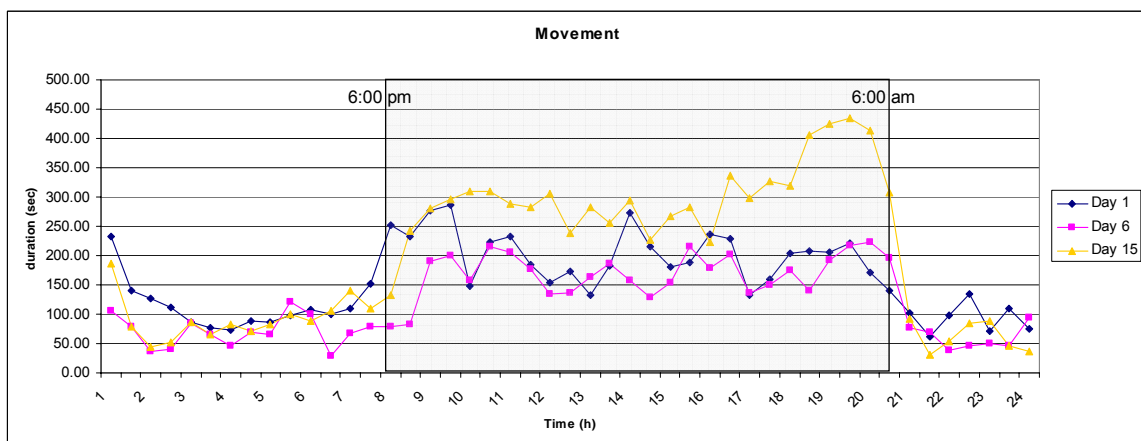
**Graph 1:** Mean distance moved each 30 minutes to follow the trend of the whole 24 hours period.

The analysis of movement which represent the mean duration of moving of rats during the 24 hours period showed a significant increase of this parameter on Day 15 when compared both with Day 1 and Day6, but the mean values of this parameter were no significantly different between Day 1 and Day 6 (Graph 3).



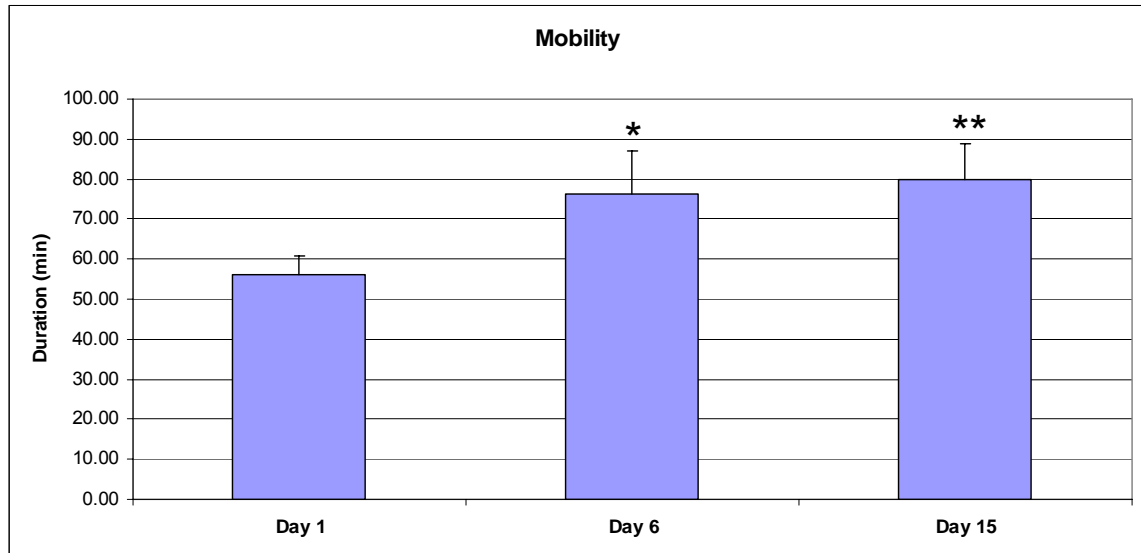
**Graph 3:** Mean duration of moving and standard errors during the whole 24 hours period.  
 \*\*  $p < 0.01$  Day 6 vs Day 15; \*\*  $p < 0.01$  Day 1 vs Day 15 (t-Student for pair-wise comparisons).

The analysis of the duration of moving every 30 minutes showed an increase of the time spent moving by animals from Day 1 to Day 15 after surgery particularly evident during the active night phase (from 6:00 pm to 6:00 am), while no significant difference was noted between Day 1 and Day 6. As the distance moved, also this parameter showed that the light-dark circadian cycle was maintained starting from Day 1 after surgery (Graph 4) and rats were active longer in terms of time during the dark phase compared with the light phase.



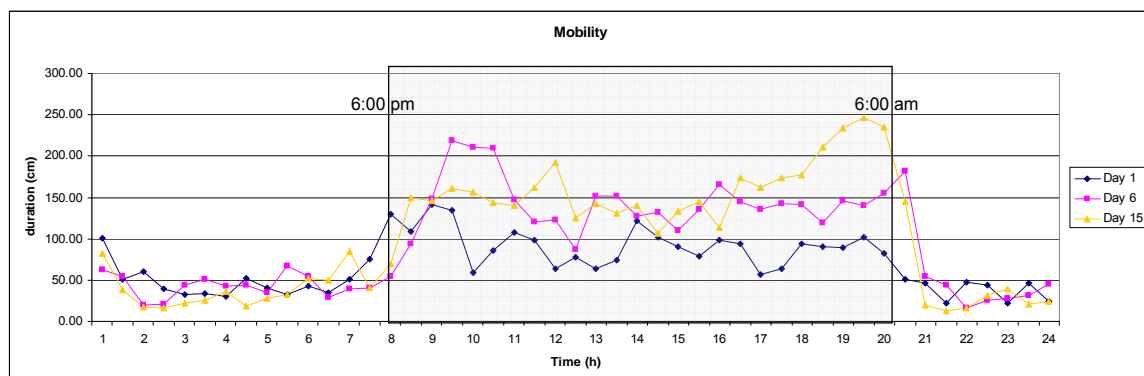
**Graph 4:** Mean time spent moving each 30 minutes.

The analysis of the parameter mobility during the 24 hours period showed a significant changes in the mean duration of active phases on both Day 6 and Day 15 compared with Day 1, when the rats were clearly less active and spent more time immobile, while no additional increase of this parameter on Day 15 compared with Day 6 (Graph 5).



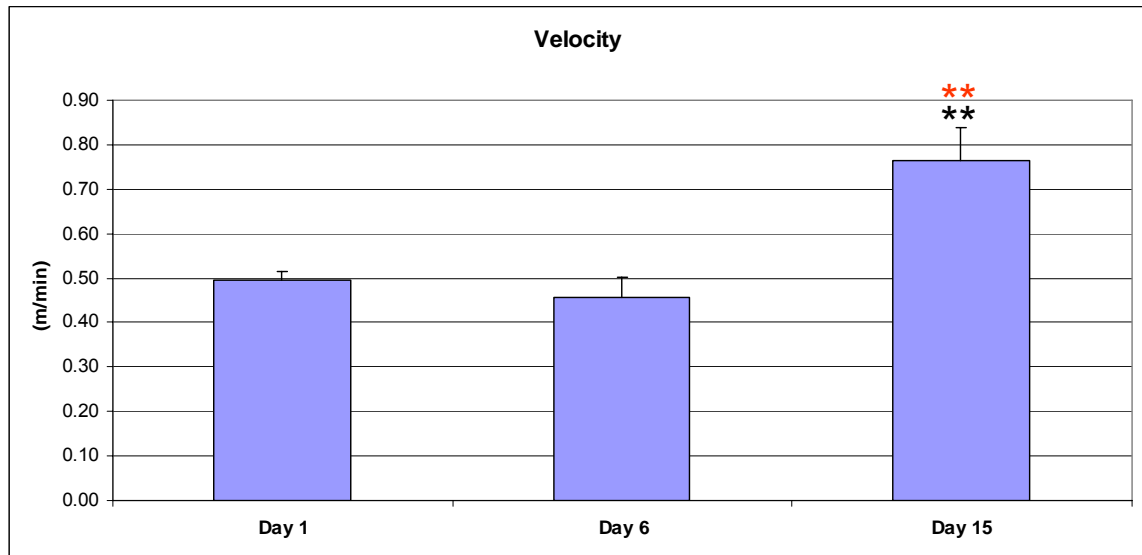
**Graph 5:** Mean time of mobile animal and standard errors during the whole 24 hours period.  
 \*  $p < 0.05$  Day 1 vs Day 6; \*\*  $p < 0.01$  Day 1 vs Day 15 (t-Student for pair-wise comparisons).

The duration of moving animals analysed in 30 minutes bins confirmed what was noted in the 24 hours period analysis (Graph 6). While the difference in the time of mobile animal between Day 1, Day 6 and Day 15 was not evident during the light-day phase, when animals were always quit and highly immobile, on the contrary during the dark-night period the animals tended to move longer and started a bigger number of mobile phases on Day 6 and Day 15. An evident pick of activity started at last hours of the night on Day 6, but more clearly on Day 15. This parameters as the previous confirmed the maintenance of the light-dark circadian cycle starting from Day 1 after surgery.



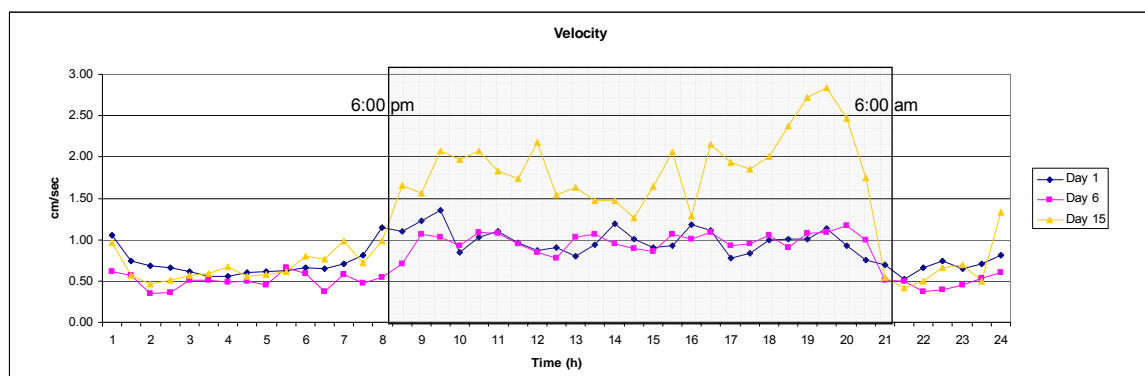
**Graph 6:** Mean time of mobile animal in 30 minutes time bins.

The mean velocity of the animals during the 24 hours period increased significantly from Day 1 to Day 15 passing from approximately 45-50 cm/min on Day 1 and Day 6 to approximately 70-75 cm/min on Day 15, without a difference between Day 1 and Day 6 (Graph 7) when the velocity remained substantially unchanged.



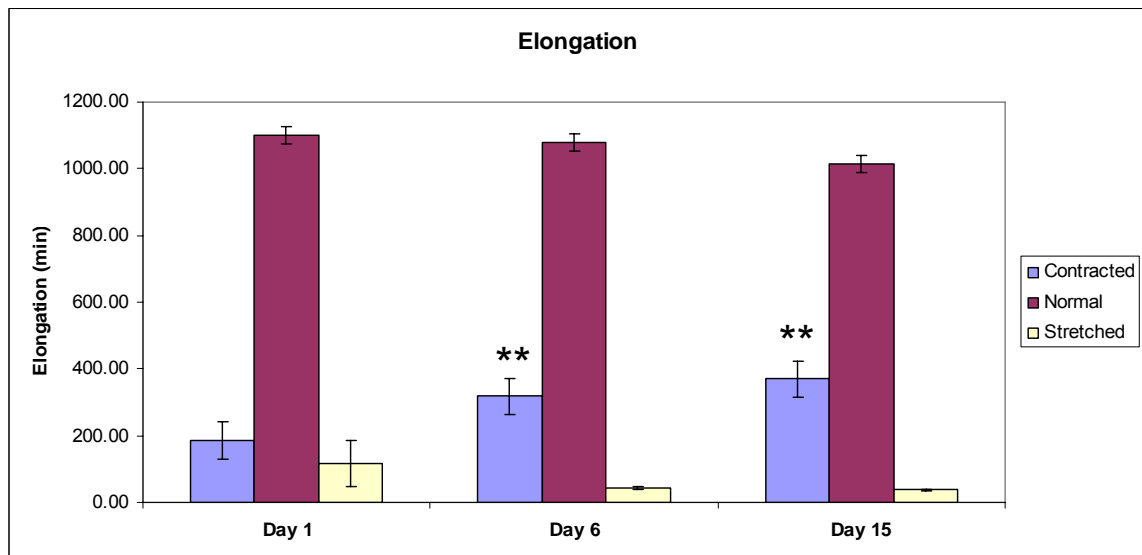
**Graph 7:** Mean velocity of animals and standard errors during the whole 24 hours period.  
 \*\*  $p < 0.01$  Day 1 vs Day 15; \*\*  $p < 0.01$  Day 6 vs Day 15 (t-Student for pair-wise comparisons).

The velocity analysed in 30 minutes time bins showed an high increase on Day 15 compared with Day 1 and Day 6, evident only during the active night phase (from 6:00 pm to 6:00 am), while no significant difference was noted between the recording days during the non-active day phase (from 6:00 am to 6:00 pm). Also this parameter clearly showed that the light-dark circadian cycle was maintained for the whole post-surgery recovery period (Graph 8).



**Graph 8:** Mean velocity of animals calculated in 30 minutes time bins.

The analysis of the parameter elongation gave information on the position maintained by the rat during the recovery period. In particular, it was noted that preferred position of animals on Day 1 post-surgery is the normal position, probably due to the abdominal pain caused by the laparotomy. The stretched position decrease from Day 1 with an associated significant increase of the contracted position on Day 6 and Day 15, with no difference between these two days (Graph 9).

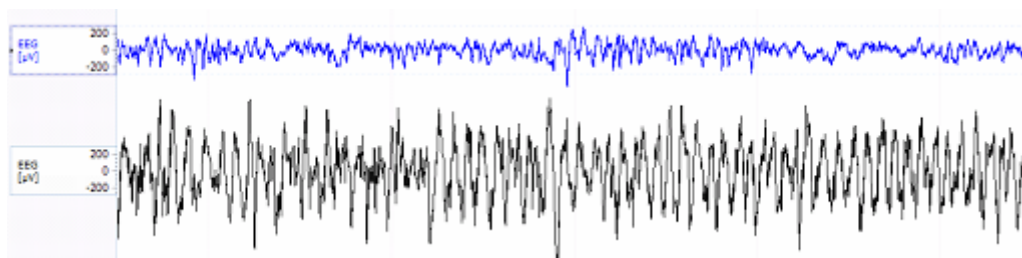


**Graph 9:** Times spent by the animal in different positions (contracted, normal and stretched) during the whole 24 hours period.

\*\*  $p < 0.01$  Day 1 vs Day 6 and Day 15 (t-Student for pair-wise comparisons).

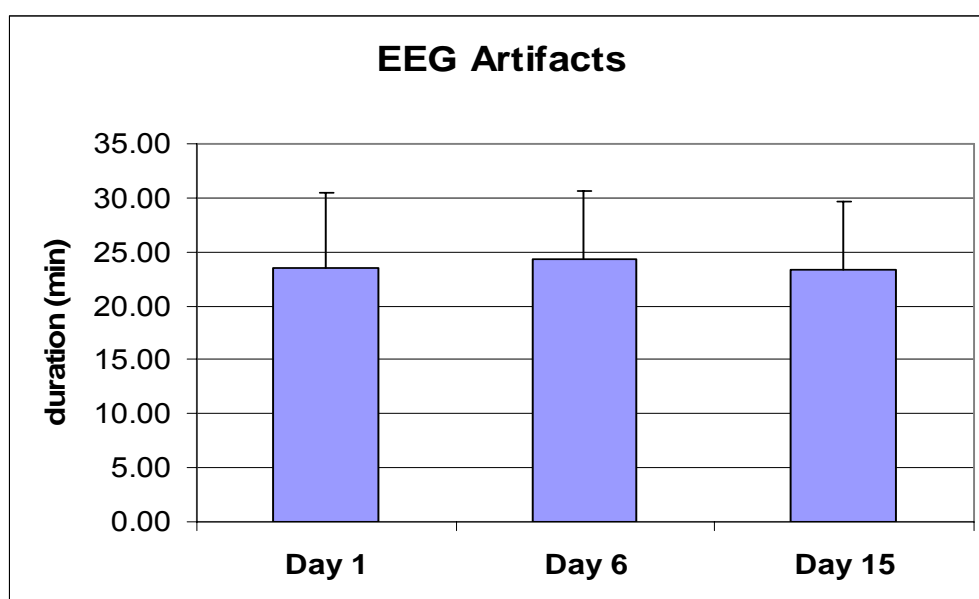
### Analysis of the Telemetric traces

The quality of the EEGs signals on Day 15 was satisfactory both for the cortical EEG (channel 3) and the hippocampus EEG (channel 2), on which the prevalent Theta activity (3.5-7.5 Hz) is clearly identifiable (Figure 22).



**Figure 22:** The telemetric EEG traces recorded on Day 15 after surgery; in blue is the cortical EEG, in black the hippocampus EEG.

During the recording 24 hours period the automatic research of artifacts on the telemetric traces (EEGs and EMG) using the Neuroscore software showed no difference in terms of duration of bad signals between Day 1, Day 6 and Day 15 (Graph 10). Approximately 20-25 minutes of the whole recording period were not readable and analyzable.



**Graph 10:** Times of artifacts on the telemetric signals (EEGs and EMG) during the recording 24 hours period.

During the manual review of telemetric traces was noted that on Day 1 the EMG signal appeared clearly disturbed by the electrocardiographic (ECG) signal, both during the Sleep (Slow Wave Sleep) and the REM or Paradoxical Sleep (Figure 23-25). These electrical alterations were not still present on the EMG trace on Day 15 after surgery (Figure 24-26).



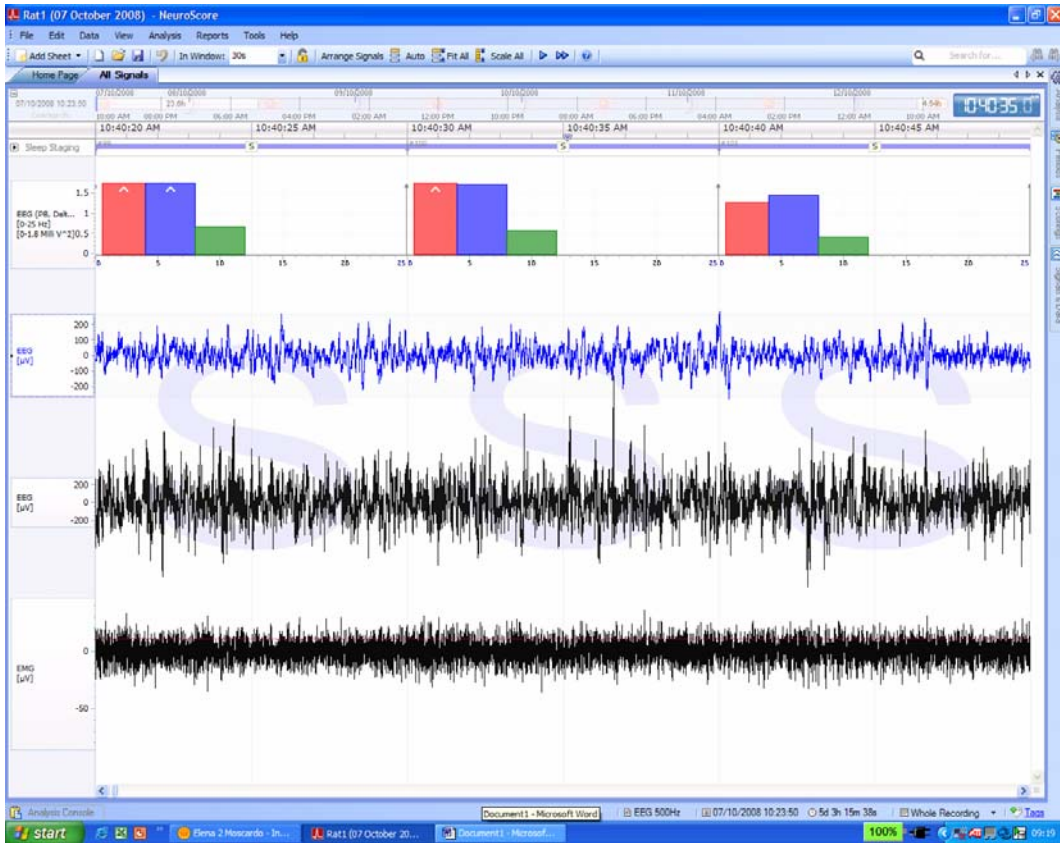


Figure 23: Day 1, telemetric traces during SLEEP stages with an evidently disturbed EMG signal.

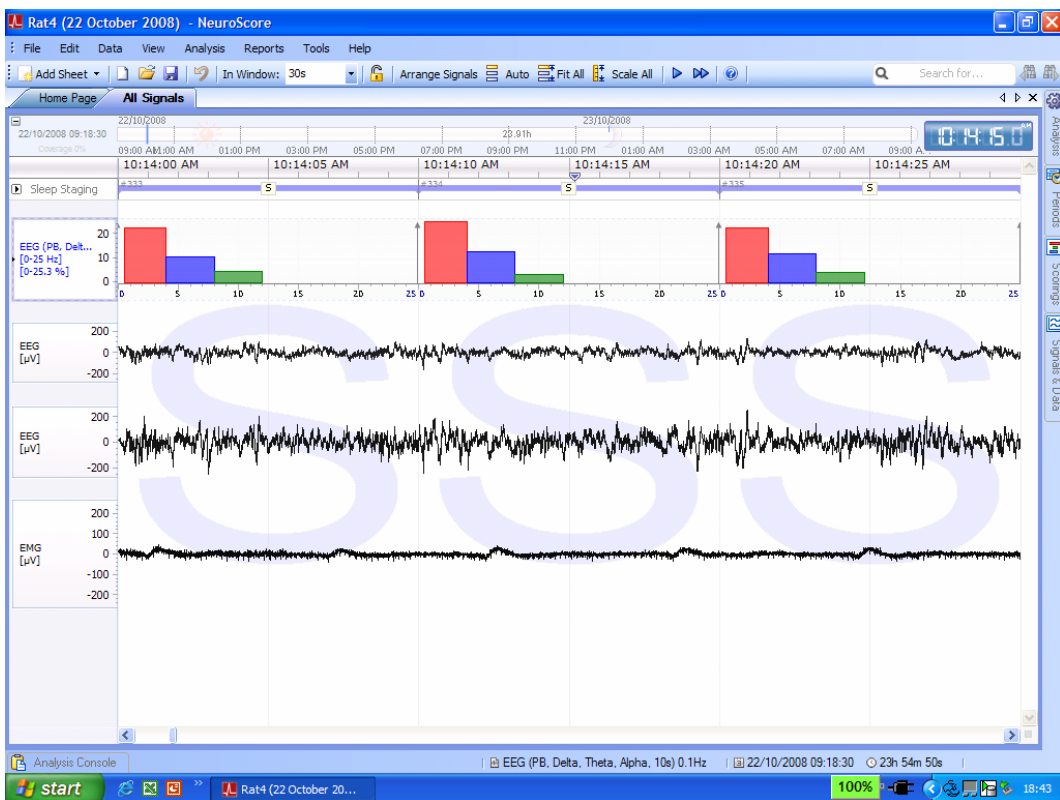
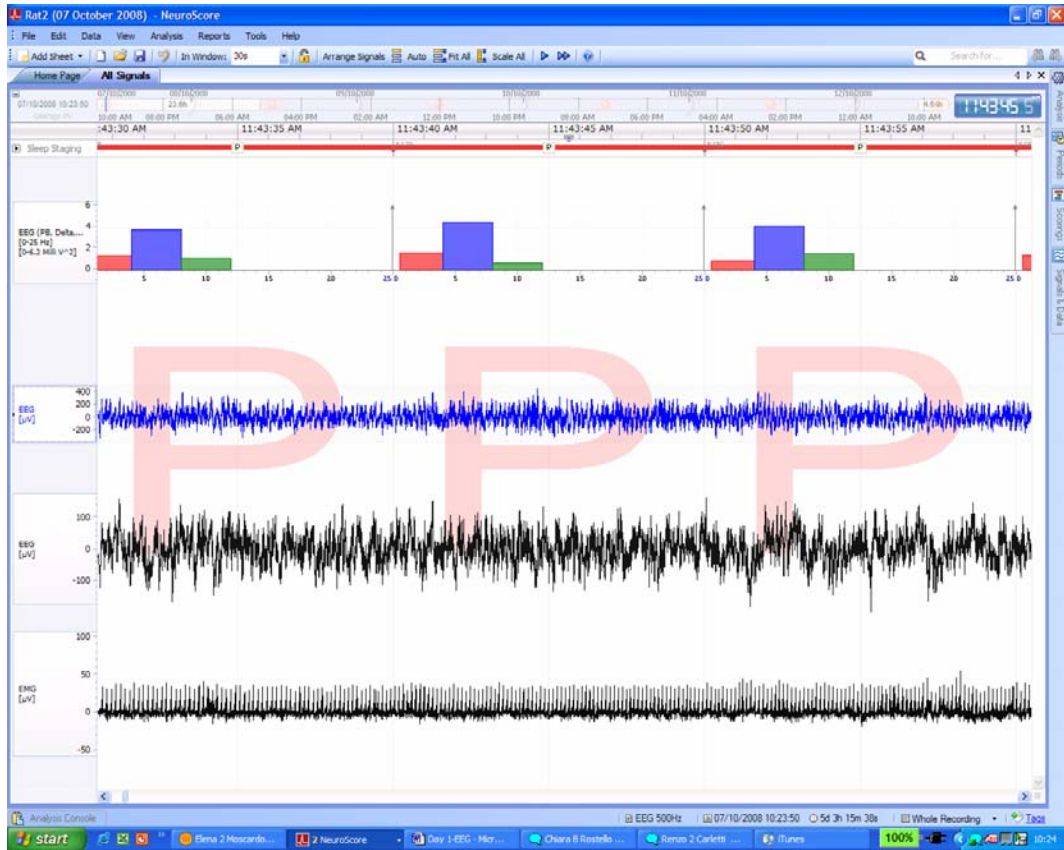
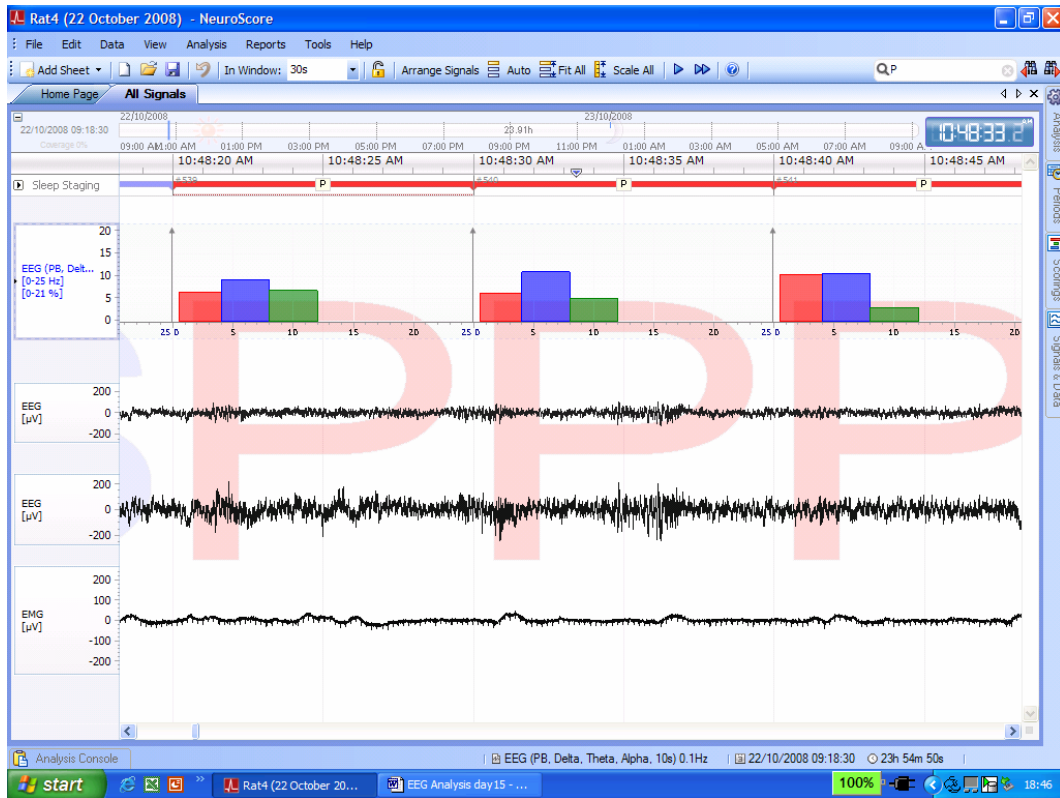


Figure 24: Day 15, telemetric traces during SLEEP stages with a good quality of EMG signal.

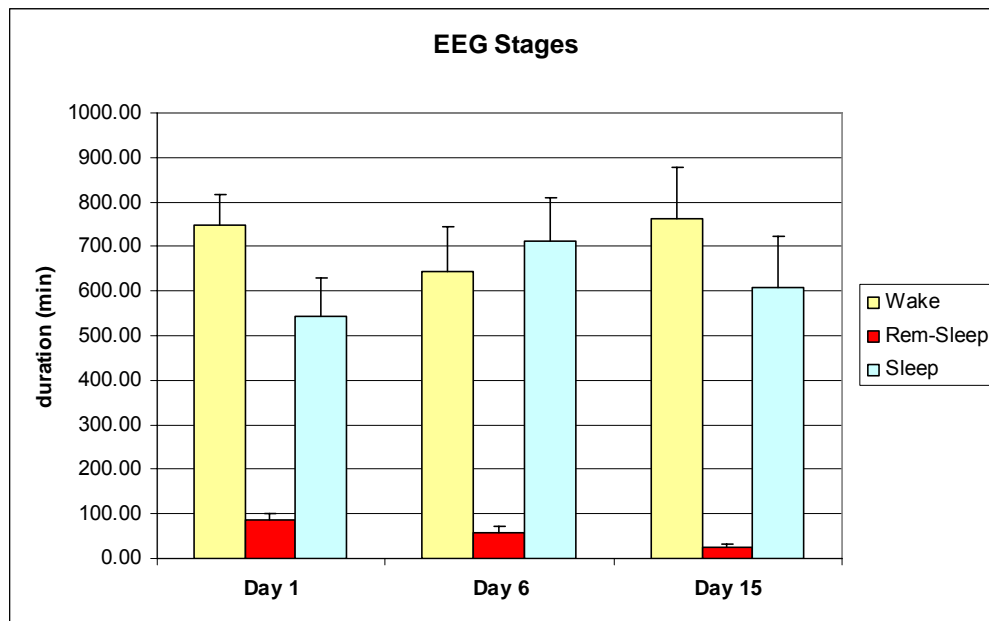


**Figure 25:** Day 1, telemetric traces during Rem-SLEEP stages with an EMG signal clearly influenced by the ECG signal.



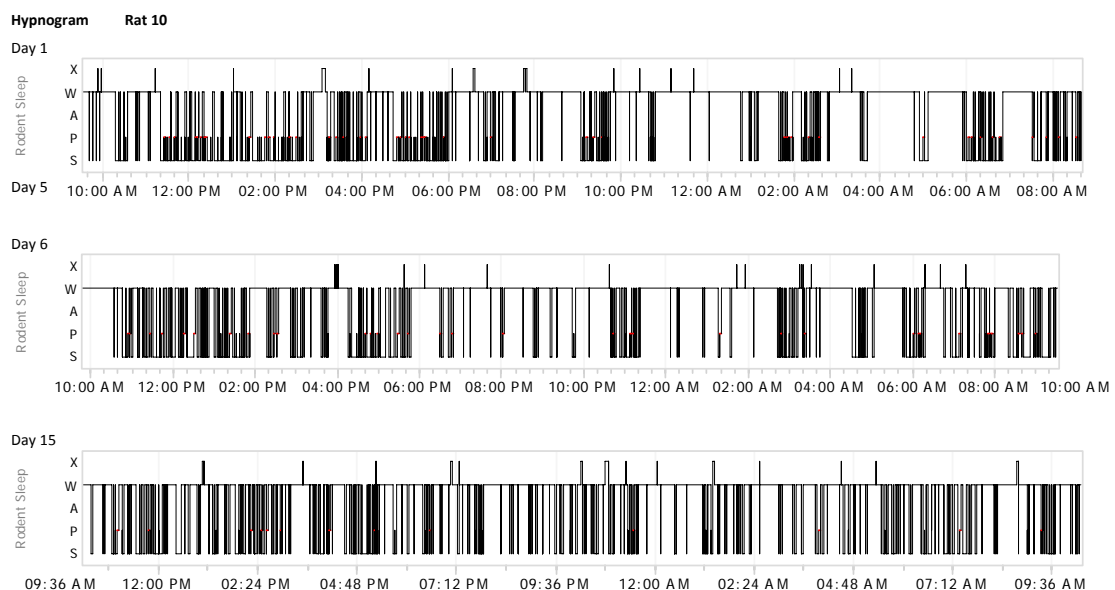
**Figure 26:** Day 15, telemetric traces during Rem-SLEEP stages with an EMG signal clearly influenced by the ECG signal.

The analysis of the three EEG stages – wake, sleep and REM or paradoxical sleep – on the whole 24 hours recording period of telemetric traces, showed no significant changes between Day 1, Day 6 and Day15 (Graph 11). In a day-time the duration of the wake-active and sleep-inactive stages were similar on each of the recording occasions, with approximately 12 hours for sleeping and 12 hours dedicated to be awake, and approximately from 25 to 90 minutes in REM-sleep.



**Graph 11:** Times spent by the animal in different EEG stages (wake, REM-sleep and sleep) during the whole 24 hours period.

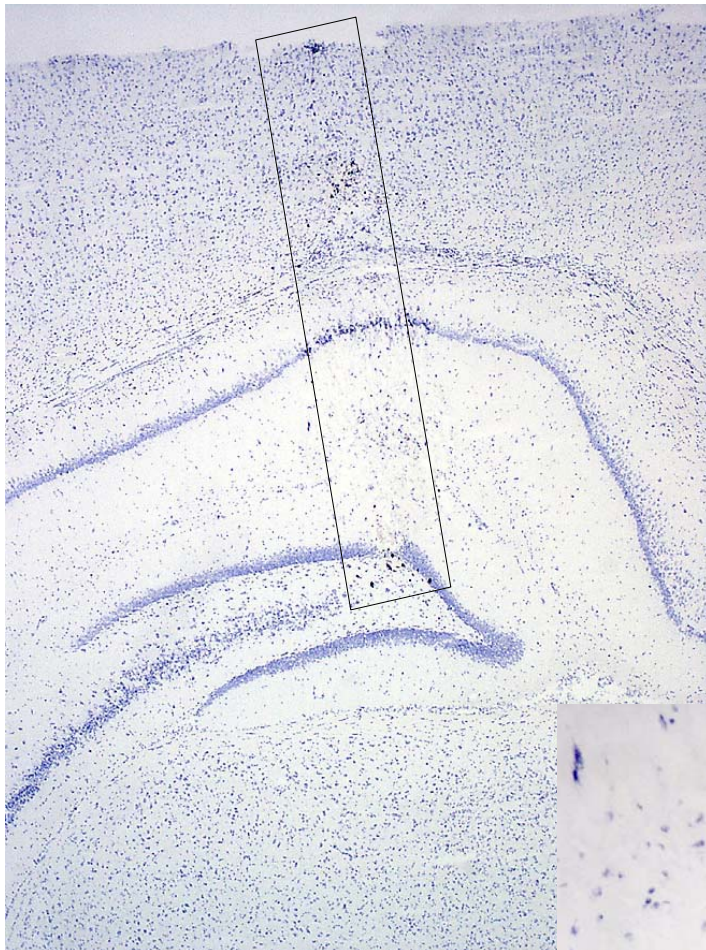
Hypnograms confirmed that the light-dark circadian cycle was maintained for the whole post-surgery recovery period, without marked differences from Day 1 to Day 15.



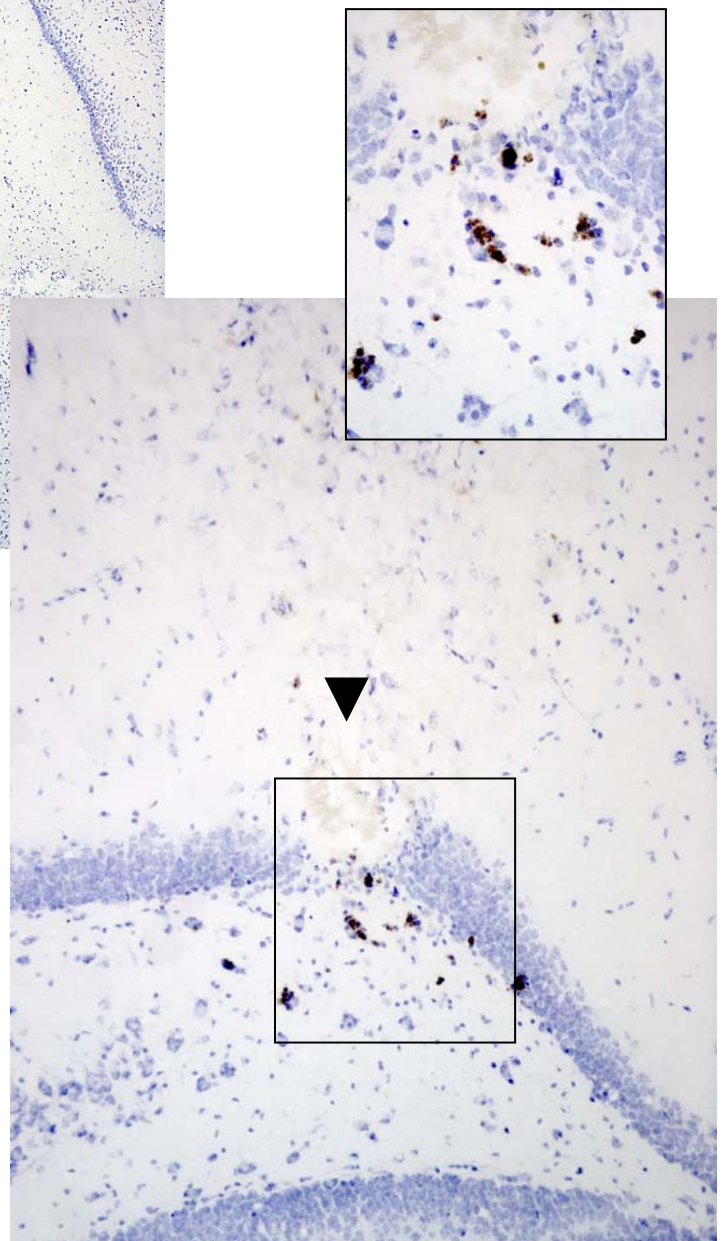


### ***Post mortem analysis***

Nissl-stained histological sections of brains showed that the deep electrode was correctly positioned as desired into the *dentate gyrus* of the left hippocampus (Figure 27-28).



**Figure 27:** The trace left by the deep electrode from the cortex down to the neurons of the *dentate gyrus* of the left hippocampus (Nissl, 2.5X).



**Figure 28:** A particular on the position of the electrode's active tip (Nissl, 10X and 40X).

## ***Discussion***

The surgical technique that was developed and applied to rats to record simultaneously cortical and hippocampus EEGs required 15 days for the complete recovery of animals both in terms of health status and quality of telemetric signals.

From the behavioural-motor activities analysis using the video data also appeared that 15 days is an appropriate period for the complete recovery of the rat from the implantation of DSI transmitters using the selected surgical technique. During the post-surgery recovery period increases of duration of moving, time of mobile animal and velocity were noted and were significant from Day 6 or Day 15. For the most of these parameters there were no significant differences between Day 6 and Day 15 as mean values calculated on the whole 24 hours recording period, but their increased values were notable during the dark-active phase (from 6:00 pm to 6:00 am). On Day 6, but more clearly on Day 15, a rapid increase of motor-activities was recorded starting at last hours of the night. These results indicate a gradual and rapid recovery of animals after the telemetric device implantation, with no major alteration of the physiological behavioural-motor activities, which are a good index of the health conditions.

The position maintained by the rat during the recovery period changed from Day 1 to Day 6 and Day 15. On Day 1 animals had preferred stretched position, probably due to the abdominal pain caused by the laparotomy. On Day 6 and Day 15 animals spent more time in the physiological contracted and normal positions, which should demonstrate the full recovery from the surgical pain.

Using a mechanical connection between the deep electrode and the DSI electrode, the telemetric recording system is able to acquire EEGs and EMG signals of good quality after 15 days from the surgical implantation. After this period of stabilisation, the system also clearly discriminated the differences between the cortical and the hippocampus traces. In fact, in the hippocampus EEG was clearly identifiable the prevalent Theta activity (3.5-7.5 Hz) of neurons of this area of the brain. The cortical EEG could be used for an appropriate automatic analysis using the Neuroscore software, with approximately 4% of the whole recording trace unreadable due to electrical artefacts, starting from Day 1. The EMG signal, used by the software for the automatic analysis, was clearly influenced by the ECG signal on Day 1, and this disturbance was still present on Day 6, even if with a lower magnitude. Therefore, based on the results of this research in order to have an easier and more accurate detection of EEGs stages, it is suggested to perform the experimental activities, record and analyse of telemetric traces from Day 15 post-surgery.

For all the post-surgery recovery period, on the whole 24 hours animals dedicated approximately 12 hours for sleeping, 12 hours to be awake and approximately from 25 to 90 minutes in REM-sleep. The three EEG stages time durations showed no significant changes between Day 1, Day 6 and Day15.

All the videos (behavioural-motor activities) and telemetric (hypnogram) parameters recorded during the recovery period showed and confirmed each other the maintenance of the light-dark circadian cycle of animals, which is present on Day 1 just after surgery, with no substantial changes until Day 15 post-surgery.

These results confirm that the applied surgical technique for the implantation of telemetric transmitter type TL10M3-F50-EEE for the simultaneous recording of cortical, hippocampus electroencephalograms and electromiogram in freely moving rats, allows a gradual, but rapid recovery of animals within 15 days. The fact that during all this recovery period there were no changes of the light-dark circadian cycle of animals, confirm the good quality of the technique and its possible application in different research areas to obtain simultaneously good physiological and behavioural-motor activities data.

Post-mortem verification of the position of the deep electrode confirmed that following the selected stereotaxic coordinates, the active-recording tip of the electrode was correctly positioned in the dentate gyrus cells layer.

**PART III:**  
**Pharmacological Validation**

## ***Methods and Materials***

### **Reference compounds and doses rationale**

Three pro-convulsing reference compounds were preliminary tested on non-telemetric rats in order to define the doses of Pentylentetrazole (GABA<sub>A</sub> receptor antagonist), 4-Aminopyridine (a K<sup>+</sup> receptor antagonist) and of Imipenem/cilastatin (a carbapenem antibiotic) causing convulsions in the male CD<sup>TM</sup> rats approximately 12 weeks old and to analyze clinical and behavioural signs preceding and accompanying the convulsive episode. These doses were selected as the most appropriate doses to be administered in the pro-convulsing validation studies using implanted telemetric animals.

### **Pentylentetrazole (GABA<sub>A</sub> receptor antagonist)**

Pentylentetrazole (GABA<sub>A</sub> receptor antagonist) from Sigma-Aldrich (Milano, Italy) was administered intraperitoneally, because this is one of the most widely used routes for administration of this pro-convulsive compound to laboratory animals in the literature (Nehlig A. and Vasconcelos A.P., 1996, Van Camp N. et al, 2003, Arrieta O. et al., 2005). For selecting appropriate doses of PTZ, it was taken into account that the susceptibility to PTZ-induced seizures is age-dependent (Klioueva et al., 2001; Veliskova et al., 1996) and the telemetered implanted CD<sup>TM</sup> rats involved in this study were 7-9 months old.

In previous investigative studies, PTZ was administered intraperitoneally to male CD<sup>TM</sup> rats of approximately 12 weeks, at 100, 80, 50, 30, 25, 10 and 5 mg/kg. The dose of 100 mg/kg induced clonic-tonic convulsions which resulted in the death of animals. Doses of 80 and 50 mg/kg caused clinical signs, such as subdued behaviour, walking in a flatted position, half-closed eyes and piloerection up to 2-3 hours post-dose, while no behavioural changes were noted at doses  $\leq$  30 mg/kg.

Based on these results doses of 10, 20 and 40 mg/kg of PTZ were used to treat intraperitoneally 6 telemetered implanted rats at a dose volume of 10 mL/kg. As vehicle 0.9% w/v Sodium Chloride was used. The low, intermediate and high doses were selected to provide from none to subconvulsive effects-signs until the development of a single short and recoverable generalized tonic-clonic convulsion.



#### **4-Aminopyridine (K<sup>+</sup> receptor antagonist)**

The 4-aminopyridine (K<sup>+</sup> receptor antagonist) from Sigma-Aldrich (Milano, Italy) was administered intraperitoneally, because this is one of the most widely used routes for administration of this pro-convulsive compound to laboratory animals in the literature (Mihály A. et al., 2001, Fragoso-Veloz J. et Tapia R., 1992, Fabene P. et al., 2006).

In previous investigative studies, 4-AP was administered to male CD<sup>TM</sup> rats of approximately 12 weeks, intraperitoneally at 0.3, 1, 3, 5, 10 or 20 mg/kg. Doses  $\geq$  5 mg/kg induced clonic-tonic convulsion which resulted in the death of animals. The dose of 3 mg/kg caused clinical signs such as all body tremors, jerks, tiptoe walking and a slight subdued behaviour from 2 hours up to 4 or 6 hours post-dose, when all these clinical signs were completely recovered. No changes related to 4-AP administration were noted at  $\leq$  1 mg/kg.

Based on these results doses of 1, 2.5 and 4 mg/kg of 4-AP were used to treat intraperitoneally 6 telemetered implanted rats at a dose volume of 10 mL/kg. As vehicle 0.9% w/v Sodium Chloride was used. The low, intermediate and high doses were selected to provide from none to subconvulsive effects-signs until the development of a single short and recoverable generalized tonic-clonic convulsion.

#### **Imipenem/cilastatin**

IMIPEM<sup>TM</sup> from Neopharmed (500mg/500mg i.m.) (imipenem monohydrate/cilastatin sodium salts), is the prototype of the carbapenem antibiotics class (imipenem) administered in combination with the renal dipeptidase inhibitor cilastatin. This reference compound was administered intravenously (bolus injection) because it was reported that in intravenous (IV) injection studies the compound produced seizure discharged on electroencephalogram (EEG) accompanied with behavioural clonic convulsions in rats (Williams et al., 1988, Dupuis et al., 2001, Horiuchi M. et al., 2006).

In previous investigative studies, imipenem/cilastatin was administered to male CD<sup>TM</sup> rats of approximately 12 weeks, intravenously at 200/200, 400/400, 750/750 and 800/800 mg/kg. The doses of 750/750 and 800/800 mg/kg Imipenem/cilastatin caused repetitive, severe and long lasting episodes of clonic-tonic convulsions. The dose of 700 mg/kg caused evident and characteristic clinical signs (i.e., alertness, awareness, tiptoe

gait, piloerection, tremors, twitches, wet dog shakes and ears flicking). No changes related to Imipenem/cilastatin administration were noted at  $\leq 400/400$  mg/kg.

Based on these results doses of 100/100, 400/400 and 700/700 mg/kg of imipenem/cilastatin were used to treat intravenously 6 telemetered implanted rats at a dose volume of 10 mL/kg. As vehicle 0.9% w/v Sodium Chloride was used. The low, intermediate and high doses were selected to provide from none to subconvulsive effects-signs until the development of a single short and recoverable generalized tonic-clonic convulsion.

### **Animals and Housing**

Male CD<sup>TM</sup> rats (CrI:CD (SD) IGS BR) supplied by Charles River Italia, Calco (LC), Italy were used. In the study with PTZ, animals had approx. 7-9 months age on the day of first dose-administration and a body weight of approx. 550 to 650 grams. In the studies with 4-AP and Imipenem/cilastatin, animals had approx. 11-12 weeks age on the day of first dose-administration and a body weight of approx. 450 to 550 grams. An acclimatisation period of 5 days before surgery was applied when animals were housed in groups of 4 into solid bottomed plastic cages with. They were housed into solid bottomed plastic cages with sawdust litter during the not recording days and into PhenoTyper cages with clean absorbent tissue (Cellu Dri Soft) during the days of the recording of data.

The cortical and hippocampal electroencephalogram, the electromyogram EMG and general activity by telemetry and the general activity by videos were recorded on the day of treatment starting from approximately 30 minutes before dosing and continuously until 24 hours post-dose.

### **Study design**

For each study using one of the three selected reference compounds, six stock animals chronically implanted with a three bio-potential transmitter (TL10M3-F50-EEE) were selected, on the basis of their clinical conditions, the quality of telemetric signals and general behaviour.

Animal numbers used for the study with PTZ are indicated below:

<b>Animal no.</b>	<b>Date of arrival</b>	<b>Date of surgical preparation</b>	<b>Transmitter S/N</b>
1	09 Jan 2008 (VR0800002)	14 Jan 2008	39089
2	09 Jan 2008 (VR0800002)	15 Jan 2008	28766
3	09 Jan 2008 (VR0800002)	14 Jan 2008	37788
4	19 Mar 2008 (VR0800189)	26 Mar 2008	25349
5	19 Mar 2008 (VR0800189)	27 Mar 2008	25220
6	19 Mar 2008 (VR0800189)	27 Mar 2008	37932

Animal numbers used for the study with 4-AP are indicated below:

7	01 Oct 2008 (VR-0800549)	6 Oct 2008	10254
8	01 Oct 2008 (VR-0800549)	6 Oct 2008	27000
9	01 Oct 2008 (VR-0800549)	7 Oct 2008	28873
10	01 Oct 2008 (VR-0800549)	8 Oct 2008	13543
11	01 Oct 2008 (VR-0800549)	8 Oct 2008	34476
12	01 Oct 2008 (VR-0800549)	9 Oct 2008	47488

Animal numbers used for the study with imipenem/cilastatin are indicated below:

13	01 Oct 2008 (VR-0800549)	6 Oct 2008	10736
14	01 Oct 2008 (VR-0800549)	7 Oct 2008	28078
15	01 Oct 2008 (VR-0800549)	7 Oct 2008	40612
16	01 Oct 2008 (VR-0800549)	8 Oct 2008	32165
17	01 Oct 2008 (VR-0800549)	9 Oct 2008	23812
18	01 Oct 2008 (VR-0800549)	9 Oct 2008	10335

A rising-dose regimen was followed for the administration of the three reference compounds as follow:

<b>Treatment</b>	<b>1<sup>st</sup></b>	<b>2<sup>nd</sup></b>	<b>3<sup>rd</sup></b>	<b>4<sup>th</sup></b>
<b>Rat 1</b>	Vehicle	Low dose	Mid dose	High dose
<b>Rat 2</b>	Vehicle	Low dose	Mid dose	High dose
<b>Rat 3</b>	Vehicle	Low dose	Mid dose	High dose
<b>Rat 4</b>	Vehicle	Low dose	Mid dose	High dose
<b>Rat 5</b>	Vehicle	Low dose	Mid dose	High dose
<b>Rat 6</b>	Vehicle	Low dose	Mid dose	High dose

Each treatment was performed weekly after a washout period of 7 days and all animals received vehicle and the three doses.

On each occasion data acquisition started at approximately 8:30-9:00 am and dosing started at 9.30-10:00 am.

### **Clinical observations and Body weights**

Animals were subjected to clinical check at least once daily. Clinical observations were performed on each dosing occasion, before dosing (BD), immediately after dosing (IAD), continuously for the first hour post-dose and approximately at 2, 4, 6 and 24 hours after dosing (end of telemetric and video recording).

The body weights were collected on each day of dosing, prior to treatment, in order to adjust the dose to be administered.

### **Telemetric measurements and video recordings**

On each day of treatment telemetric signals (EMG, cortical EEG and hippocampal EEG traces) and videos were recorded simultaneously for approximately 1 hour before dosing and 24 hours post-dose, in order to analyse and classify in details any behavioural and EEG change noted.

Telemetric and video recordings were made with the animals singly housed in their PhenoLab cages and all the EEG-video-Telemetry system previously described (Part I) was used.

## ***Results and Discussion***

A different clinical and behavioural appearance of the convulsion in PTZ, 4-AP and Imipenem/cilastatin treated animals was noted from the analysis of all clinical observations collected.

In particular, PTZ at 50 mg/kg caused a single generalised tonic-clonic convulsion in 2/4 rats, completely recovered within 24 hours post-dosing. At lower doses (25 and 5 mg/kg) were observed dose-related in severity clinical signs (i.e., half-closed eyes, subdued behaviour, piloerection, walking in flattened position) between 5-10 minutes and 2 hours post-dosing.

The doses of 3, 1 and 0.3 mg/kg 4-AP caused dose-related in severity clinical signs (i.e., alertness, awareness, tiptoe gait, piloerection, tremors, twitches) between 30-40 minutes and 4 hours post-dosing.

The doses of 750 and 800 mg/kg Imipenem/cilastatin caused repetitive, severe and long lasting episodes of clonic-tonic convulsions. The dose of 700 mg/kg caused evident and characteristic clinical signs (i.e., alertness, awareness, tiptoe gait, piloerection, tremors, twitches, wet dog shakes and ears flicking).

This information is used to select the appropriate doses of PTZ, 4-AP and Imipenem/cilastatin to be administered to implanted telemetric rats in the pro-convulsing validation studies. The analysis of telemetric data and videos collected from these treated telemetric rats (6 for each reference compounds) is currently ongoing.

## Final Remarks and Conclusions

*All the experiments described into this research work were conducted in the Research and Development Centre of GlaxoSmithKline Verona, Italy, in the Laboratories of Safety Pharmacology Group within the Safety Assessment Department. All experiments were carried out in accordance with Italian regulation governing animal welfare and protection and the European Directive 86/609/EEC, and according to internal GlaxoSmithKline Committee on Animal Research & Ethics (CARE) review.*

These results of this research confirm that the settled-up integrated video-telemetric system (Part I) and the applied surgical technique for the implantation of telemetric transmitter type TL10M3-F50-EEE (Part II) for the simultaneous and synchronised recording of cortical, hippocampus electroencephalograms and electromiogram in freely moving rats, can find appropriate and innovative applications in different research areas, where is of particular interest to obtain simultaneously physiological and behavioural data.

In particular, the EEG-video-telemetry system could be applied in Safety Pharmacology testing for assessing central nervous system (CNS) side effects.

The first interesting application in this area of safety, is that related to the analysis of the relationship between seizures (EEGs traces) and convulsions, where the model could contribute in refining the conventional models (i.e., tests of chemical and electrical induction of seizures) to assess the pro-convulsive potential risk of New Chemical Entities (NCEs), but also in the behavioural characterisation of seizure activities of brain.

This application was only started during this research (Part III), when the EEG-Video-Telemetry system was used to investigate physiological and behavioral correlations following administration of pro-convulsive reference compounds (i.e. Pentylentetrazole, 4-Aminopyridine and Imipenem/cilastatin) in rats. In this area the system should allow the identification of the seizure-inducing potential, until convulsive threshold doses and the establishment of relationship between behavioral and clinical signs preceding and accompanying convulsive episodes and paroxysmal activity of the brain.

A second future possible application of the EEG-video-telemetry system in the CNS-safety, is that related to the analysis of the relationship between EEGs traces and behavioral patterns, where the model could contribute in assessing specific CNS side

effects such as drug related sleep disruption, impairments on learning and memory, visual and auditory functions alterations.

The system should give to researchers lots of ideas for innovative possible applications also in routine research areas. For example it could be used for analyzing the influence of post-operative care procedures, housing environmental conditions and training procedures on the experimental collected data and results.

Telemetric waveforms and the activities of animals collected with this integrated system provide unprecedented insight into the correlations between phenotypical-behavioural and electrical brain activities. The system should reduce the number of animals used, improve/refine the data quality by maximizing the amount of information gained from each experimental animal and reduce animal stress and discomfort (3Rs).

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## ***Ringraziamenti***

Questo lavoro di ricerca non avrebbe mai potuto essere realizzato se la mia amatissima famiglia, mio marito Alessandro e i miei due figli Francesca e Michele, non mi avessero concesso per lavorarci parte del tempo che doveva essere a loro riservato, e non avessero pazientemente sopportato i miei malumori e momenti di nervosismo conseguenti alle notti quasi insonni passate a pensare e a lavorare a questo progetto.

Uno speciale ringraziamento pieno di affetto va alla mia amica e collega la Dott.ssa Chiara Rostello, che mi ha affiancata costantemente ed infaticabilmente nella valutazione dei disegni sperimentali, nelle attività pratiche e nella elaborazione dei dati sperimentali, sempre dandomi la sua opinione sincera e professionale e mettendo spesso in luce i lati a volte troppo fantasiosi ed irrealizzabili delle mie ipotesi di ricerca.

Grazie al mio collega Luciano Cunego per l'insostituibile e brillante capacità di aiutarmi in tutto quello che ha a che fare con la tecnologia – dal cavo elettrico giusto che è sempre capace di trovare, al computer più complesso che per lui non ha segreti. Sono molto grata ad Eric Rieux e Marco Boscaro di Data Science International e a Reinko Roelofs di Noldus Information Thecnology per il valido supporto tecnico datomi nel superamento rapido e proattivo dei problemi per arrivare da un'idea iniziale alla realizzazione concreta di un sistema complesso, capace di integrare la telemetria con l'acquisizione ed elebarozazione delle immagini.

Non posso dimenticare di ringraziare il Dott. Renzo Carletti per i concetti di neuroanatomia che mi ha insegnato e per l'aiuto datomi nella reaizzazione dei preparati istologici, colorazioni e fotografie.

Grazie a Devid Sokal per l'aiuto datomi nell'apprendimento dei rudimenti della tecnica di impianto di elettrodi cerebrali profondi, e a Francesco Crespi per la metodologia di indagine post-mortem del loro corretto posizionamento.

Infine grazie a Eddie Vedder dei Pearl Jam, che con la sua straordinaria voce mi ha accompagnato con le canzoni dell'album "Into the Wild" durante l'elaborazione dei dati, che spesso ha richiesto lunghe ore passate davanti allo schermo del mio computer e nella stesura finale di questo lavoro.