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Neuropsicologiche, Morfologiche & Motorie

DOTTORATO DI RICERCA IN
Imaging Multimodale in Biomedicina

CICLO **XXIII**

TITOLO DELLA TESI DI DOTTORATO

Video Telemetric EEG System In Physiological and Pathological Conditions

S.S.D. BIO/16 – ANATOMIA UMANA

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1. Objectives and Aim

The aim of present research activities is the application of a new Video-Telemetry EEG (Video-tEEG) 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) were investigated, in order to assess brain's activity both in physiological and pathological conditions.

The potential future applications of this Model in animal research were investigated. In particular this research had three principal objectives:

1. *Relations between behavioural patterns and EEGs traces in different environmental conditions.*

   It was evaluated how different environmental conditions (enriched or not, with olfactory stimuli or not) affect behaviour and encephalographic parameters (sleep and theta activity patterns) in rats.

2. *Relation between seizures and convulsions.*

   It was investigated the enhanced value of using this Video-tEEG Model for the pro-convulsing liability assessment following the administration of new chemical entities (NCEs). It was analyzed the relation between seizures and convulsions (EEGs traces alterations versus behavioural evidences) in rats.

3. *Video-telemetry-EEG system and a PK-PD modeling study.*

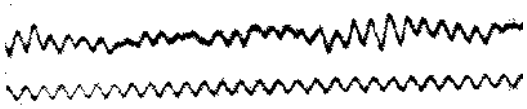
   It was evaluated the feasibility to integrate a Video-telemetry Electroencephalography (video-tEEG) system with a set up for sequential blood bleeding in the laboratory rat.

2. Introduction

The behaviour is considered the ultimate integration of nervous system functions at the level of the intact organism (Mello NK, 1975; Vanderschuren LJ et al., 1997), while the physiology gives insight into phenomena that are hard to see by a simple observation.
The studying of the combination between physiology and behaviour is often a very complex field (Santiago Ramon y Cajal, 1897) but it allows a complete and coherent assessment of nervous system functionalities and alterations.
An integrated acquisition and analysis of EEGs physiological traces and the behaviour in freely moving animals should be used to assess simultaneously the behavioural and the electrophysiological activities of the brain assessing Central Nervous System activities/functionalities.

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 associated to behaviours observed 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:

- the EEG picks up synchronization of neurons (amplitude of about 100 μV on the scalp and about 1-2 mV on the surface of the brain). The electrodes are not sensitive enough to pick out individual action potentials of a neuron or the electric unit of signalling in the brain. Besides the obtained data is the resulting of the electrical activity as a consequence of the release of inhibitory, excitatory or modulatory neurotransmitters;

- the EEG has limited anatomical specificity to triangulate the source of the electrical activity (side effects).

EEG advantages:

- the brain works through its electric activity and EEG is the only method to measure it directly;
- the time resolution of this technique is very high (down to sub-millisecond). Besides recent publications report integration between EEG or MEG and MRI or PET in order to get higher temporal and spatial resolution.

Seizure

A seizure is defined as a period of rhythmic, synchronized abnormal neuronal activity that may result in a number of behavioural changes and it can be detected by measuring the electrical activity of the brain. The electroencephalogram (EEG) is the gold standard for seizure detection, both in animals and humans. A convulsant compound induces overt motor effects until tonic-clonic convulsion, whereas a pro-convulsant drug increases the severity of the abnormal behavioural activity and/or the convulsion events (Easter A et al., 2009). It is important to underline that seizure activity is not always followed by the behavioural changes that define a convulsion. The EEG is particularly useful when new drugs may cause seizure activity in the brain without eliciting overt behavioural manifestations (Stanojlović O et al., 2009). In addition to electrical seizures, other abnormal waveforms on the EEG can be identified such as spike-wave discharges (SWD), polyspike-wave and trains of spikes (Lüttjohann A et al., 2009; Calvin WH, 1975).

Video Recording

The video image is the most innovative methodology, which allows capturing of the behavioural patterns of a freely moving and undisturbed animal in its environment. Video recording of a freely moving and undisturbed animal can help in the correct definition/analysis of behavioural patterns.

Dedicated software-hardware systems, using video tracking technologies, allow the quantitative and qualitative measure of the behavioural activities. EthoVision XT, by Noldus Information Technologies, is the most widely applied software platform for automated tracking and analysis of animal movement and activity (Spink AJ et al., 2001).

Radio-telemetry

The radio-telemetry is a technology of radio-signals recording from chronically implanted transmitters (Figure 1) 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 a more complex system capable of simultaneously monitoring several variables (BP, ECG or EEG, BT, LA) in large and small animals.

The use of conscious unrestrained animals implanted with telemetry transmitters that monitor physiologic parameters is well known (Kramer K and Kinter LB, 2003; Williams PA et al., 2006) and has great advantages for eliminating the influences of anaesthetic and restraint-induced stress. These factors may alter the sensitivity of the model to detect drug-induced effects. Because of these advantages the use of conscious unrestrained animals has been recommended in Safety Pharmacology studies (ICH S7A and ICH B).

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

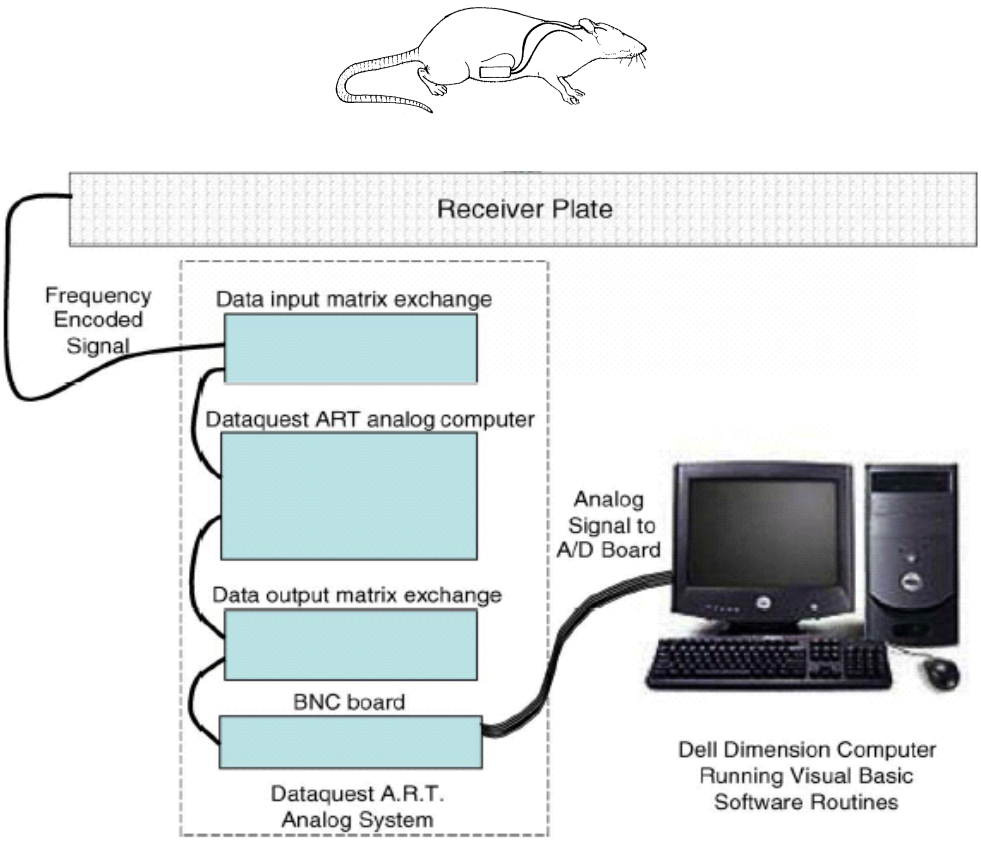


Figure 1: *A scheme of the telemetric recording system.*

Experimental challenges

Environmental enrichment

Following differential experiences provided by enriched environmental conditions, several studies have shown significant changes in laboratory animals at morphological and behavioural levels (Mora F et al., 2007; Van Praag H et al., 2000), but very few studies have investigated effects of these conditions on sleep pattern. These studies report a significant increase in the REM sleep duration in animals after incremented learning (labyrinth session or enriched conditions) with respect to the controls and no significant increase in slow-wave sleep duration (Lucero MA, 1970; Mirmiran M et al., 1982). A recent work showed that rats in unenriched cages slept less frequently and for shorter durations than rats housed in the enriched conditions (Abou-Ismail U.A. et al., 2010). These results suggest the REM phase of sleep might be involved in the processing of information acquired during wakefulness (Lucero MA, 1970; Mirmiran M et al., 1982).

In literature there are different results about exploratory and movement parameters in relation with environmental enrichment. Some studies in rats have reported an increase in exploratory (Abou-Ismail U.A. et al., 2010) and in moving behaviors (Abou-Ismail U.A. et al., 2010) in unenriched cages as compared to enriched cages; others have reported similar increase in animals housed in enriched cages (Townsend P., 1997; Orok-Edem E. and Key D., 1994) and only one paper did not report any differences between groups (Foulkes A., 1994).

Enrichment and exercise are often associated with anatomical changes of *dentate gyrus*. Some researchers have found an increase in the survival of newborn neurons in the *dentate gyrus* of adult rondents raised in an enriched environment (Van Praag H. et al., 1999; Kempermann G. et al., 1997). One of the questions that remains open is whether the effects of running and enrichment are additive.

Besides, some data suggested the correlations between cortex, hippocampal electrical activities and olfaction (Zibrowski EM and Vanderwolf CH, 1997). In addition the *dentate gyrus* seems to be crucial for new patterns of learning: its maximal activation is in response to the acquisition of novel information (Montag-Sallaz M et al., 1999).

Theta rhythm

Theta rhythm is the most prominent and most studied low-frequency oscillation in the hippocampus and in rat this rhythm is a large amplitude (1-2 mV) oscillation of 5-12 Hz (Buzsaki G, 2002). In the behaving rat, theta rhythm is dominant during walking and rapid-eye-movement sleep, while irregular slow activity predominated during immobility and

slow-wave sleep (Vanderwolf CH, 1969). It has previously been suggested that theta rhythm in the hippocampus and neighboring cortical regions may gate the flow and storage of information both within and between these areas during various forms of mnemonic processing (Chrobak JJ and Buzsaki G, 1998; Shen J et al., 1997). The theta rhythm represents a "tag" for short-term memory processing in the hippocampus (Vertes RP, 2005).

Pentylentetrazole (PTZ)

Central nervous system (CNS) safety pharmacology is mainly focussed in early identification of unwanted or adverse effects on the nervous system. In the last five years specific attention is increasing on seizure liability and on how properly and efficaciously predict this risk by a general review of all the standard and non-standard non-clinical models for investigating convulsion and pro-convulsion and their powerful integrated use in a cascade approach (Pugsley MK et al., 2008; Löscher W, 2009; Easter A et al., 2009; Valentin JP et al., 2009). Preclinical testing for convulsant and pro-convulsant activity is considered one of the most important assessments following administration of new chemical entities (NCEs), which may cause and/or promote convulsions in humans, lowering the seizure-precipitating threshold required for altering the normal balance of excitation and inhibition in the brain. In this assessment, false positive will eventually cause a potential failure of a new compound, with consequent therapeutical and economical disadvantages. On the other hand, false negative will fail to predict liability, with important and potentially dramatic ethical and, finally, economical issues. Furthermore, seizure risk assessment should be taken into account not only for CNS-targeted compounds, but also for non-CNS-targeted ones. In facts, approximately 50% of the non-CNS-targeted compounds induced electroencephalogram (EEG) pathological alterations (Easter A et al., 2009). In order to correctly identify seizure liability of NCEs it is thus required that: a) the preclinical model is as translational as possible to the human condition; b) all the clinical aspects characterizing the animal condition are known and correctly taken into account in the data analysis; c) the biomarkers involved in the pathology are fully identified and d) the techniques applied to the model are able to cover and monitor the biomarkers themselves. One of the most frequently used chemical model for assessing pro-convulsant/anti-convulsant drug activity and to identify pharmaceuticals that may alter seizure susceptibility is pentylentetrazol (PTZ) (Löscher W and Schmidt D, 1988; White HS et al., 2008). PTZ, also known as pentetrazol and metrazol, is a CNS chemical stimulant which passes easily in the blood-brain-barrier and acts predominantly

by antagonizing GABAergic inhibition effecting the picrotoxin site of the chloride ionophore of the $GABA_A$ receptor (Löscher W, 2009; Figure 2).

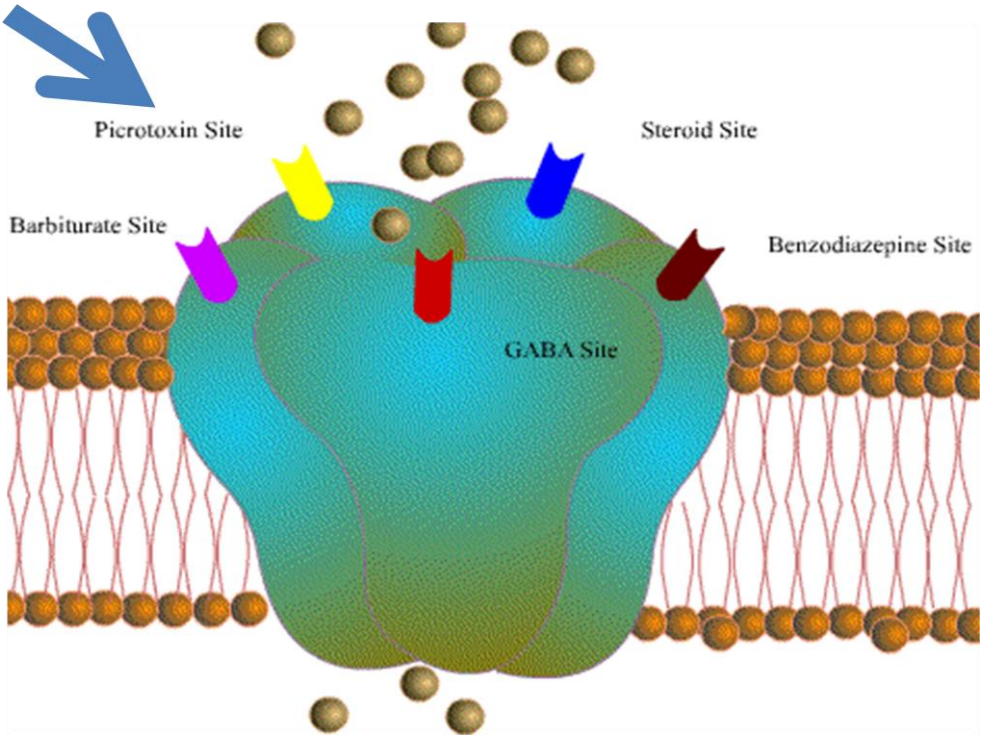


Figure 2: *A scheme of PTZ action mechanism.*

PTZ can be administered intravenously (i.v.), intraperitoneally (i.p.) or subcutaneously (s.c.), by the mean of a single injection (Beig MI et al., 2007; White HS et al., 2008; Löscher W, 2009) or by repetitive injections (Lüttjohann A et al., 2009).

At low doses (≤25 mg/Kg ip), PTZ induces seizures, which are defined as typical changes in the EEG due to rhythmic, synchronized, abnormal neuronal activity resulting in 5-7 Hz frequency (theta waves) short-lasting (2 to 10 seconds) trains of spike-and-wave complexes (Wong M et al., 2003), accompanied by freezing of the animal with rhythmic jerks of whiskers and/ or ears (Schickerová R et al., 1984). At higher (≥40 mg/Kg ip) PTZ doses, two types of motor convulsions can be elicited: a) minimal, restricted to forelimbs, and predominantly clonic; and b) major, generalized tonic-clonic responses in the form of muscle contractions of the whole body, often followed by a cramped tonic state (Kliouéva IA et al., 2001; Beig MI et al., 2007; Lüttjohann A et al., 2009).

In the last decade and mainly in the last five years EEG signals acquisition from conscious laboratory animals by fully-implantable telemetry or other recording systems has been used by many research groups not only for sleep, but also for seizures detection and

analysis (Vogel V et al., 2002; Bastlund JF et al., 2004; Bragin A et al., 2005; Authie S et al., 2009). Also the video monitoring associated with the EEG acquisition has progressively increased its relevance, because it allows to differentiate electrical artefacts from EEG seizures and to distinguish EEG seizures (non-convulsive) from those with a motor-behavioural (convulsive) component (Bastlund JF et al., 2004; Williams PA et al., 2006).

Despite the wealth of data collected in rats of different strains and ages indicating possible dissociation between EEG alterations and behavioural scoring (Pitkanen A et al., 2006; Stanojlovic O et al., 2009) and a poor correlation between EEG activity and individual phases of the motor convulsion (Schickerová R et al., 1984), both basic research and applied pharmacology are mainly using convulsions as marker of pro-convulsive or pro-epileptogenic risk. In particular, from a PubMed search it resulted that approximately 20% of the articles published in the 2009 and only approximately 11% of the total published literature from 1975 to 2009 focused on PTZ pro-convulsive risk assessment used an EEG evaluation (Figure 3), whereas, behavioural evaluation is responsible of the late identification of seizures liability (Easter A et al., 2009).

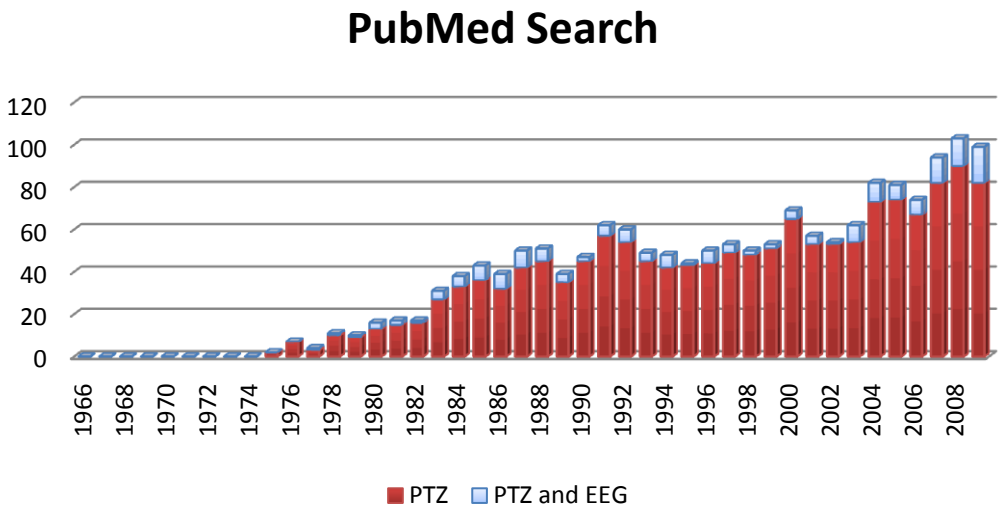


Figure 3: *PubMed Search from 1966 to 2009 done by Year[DP] ptz and Year[DP] ptz and eeg; Limits Activated: Animals and English. On a total of 1496 articles on PTZ, only 170 articles used the EEG analysis, which represents 11.4% of the total published literature on the argument.*

Chronic vascular cannulation model

The increased use of small animal models in pharmacological, biomedical and behavioural research has brought about an increased interest in techniques for vascular cannulation.
To reduce stress and release of stress-releated hormones, it is desirable to refine experimental procedures, avoiding the stress caused by handling, restraint and sampling. The use of a blood sampling device makes it possible to obtain consecutive blood samples from a conscious rat without human interference, once catheter has been installed (Royo F et al., 2004). Manual blood sampling normally results in a stress response while a blood-sampling device by catheter is able to draw blood samples without inducing a corticosterone release in blood. In fact, although the use of a chronic vascular cannulation model necessitates an invasive procedure to install the catheter, after a right recovery the blood sampling itself seems to be associated with little or no stress (Royo F et al., 2004; Gärtner K et al., 1980).

Traditional preclinical pharmacokinetic and pharmacodynamic (PKPD) studies require separate phases with multiple subject groups to characterize pharmacokinetic (PK), pharmacodynamic (PD), and toxicology endpoints of drug candidates. However, prevailing study paradigms do not take into account possible dosing variations between animals, changing environmental conditions and individual genetic variability that can alter individual responses to drug. One way of controlling for these variables and assessing true PKPD is to measure multiple parameters in the same animal.
Besides the use of a vascular cannulation and a theter device allows to monitor test compound dose-response curve avoiding stress due to handling procedure, to re-use animals, which leads to a reduction in animal use and the chance to evaluate absolute PK parameters (e.g., absolute bioavailability) and finally to apply PK/PD modelling to interpret in vivo signs in relation with test compound dose-response curve.

A potential application of this integrated video-tEEG and blood sampling system for acquisition of multiple PD endpoints with simultaneous PK sampling from the same animal could be the investigation of seizure/convulsion liability of test compounds with an enhanced translatability to human.

3. Methodology

The Video-Telemetry-EEG Model

The Video-Telemetry-EEG (Video-tEEG) Model consists of the Radio-telemetry for physiological parameters recording and the video-images technologies for video recording. 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 to apply different models such as sleep pattern and study of pro-convulsive drug activity. 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; Mirmiran M et al., 1982). Besides the possibility of distinguishing between cortical and limbic (hippocampus) epileptiform activity is considered an essential issue when the initiation and spread of seizures is under evaluation (Bastlund JF et al., 2004, White AM et al., 2006, Williams P et al., 2006).

The set up and the general evaluation of the Video-tEEG system, capable of simultaneously assessing animal behaviour (using video tracking technologies) and the electrophysiological activities (using radio-telemetry) in freely moving rats was conducted in a previous research work (Moscardo PhD Thesis, 2009; Moscardo E and Rostello C, 2010). That work was concerned to the refinement of the surgical technique as well as the integration and synchronization of the commercially available Dataquest telemetry system and the Noldus video system, in order to study cortical, hippocampal EEG waveforms in combination with behaviour and locomotion

The use of this integrated Video-tEEG system in different pre-clinical research areas could provide advantages for an ethical use of animals, and therefore some of possible applications of this system were evaluated in the present research work. The last but not least application could be the possibility to assess a system for combining Video-tEEG with a remote blood sampling device, which is considered potentially extremely helpful for a reliable pharmacokinetic/pharmacodynamic (PK/PD) modelling of behavioural and EEG changes following the administration of a new candidate drug by a time-right identification of plasma concentrations. This opportunity for application was also investigated in the present research.

Telemetric probes surgical 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_2 and was maintained throughout the surgical procedure under 1.5-2% isoflurane anaesthesia (Forane, Abbott), vaporised in 1.5-2 L/min O_2; 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 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±1°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 (Iodiopovidone 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.

Antibiotic cover and analgesic treatment

The day of surgical preparation animals received:

- Rimadyl™ (Carprofen 50 mg/mL) 5.0 mg/kg (sc), Pfizer Inc. U.S.A.; animals received one subcutaneous dose immediately before surgery;

- Rubrocillina™ Veterinaria, Nuova ICC, Italy 1.0 mL/kg (benzylpenicilline benzatinica 125,000 U.I./mL plus dihydrostreptomycin sulphate 50 mg/mL); animals received one subcutaneous dose immediately before surgery;

- Sodium Cloride 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 RubrocillinaTM Veterinaria 1.0 mL/kg (sc) and RimadylTM 5 mg/kg (sc) once daily.

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°. A deep electrode (stainless steel electrode E363/2, Plastics One) was crimped onto the positive lead (red) of channel 2 (hippocampus EEG biopotential) (Figures 4-5). Channel 1 was used for EMG recording.

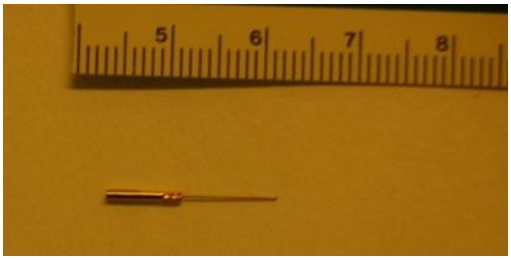


Figure 4: *The stained steel electrode used to record hippocampal EEG.*

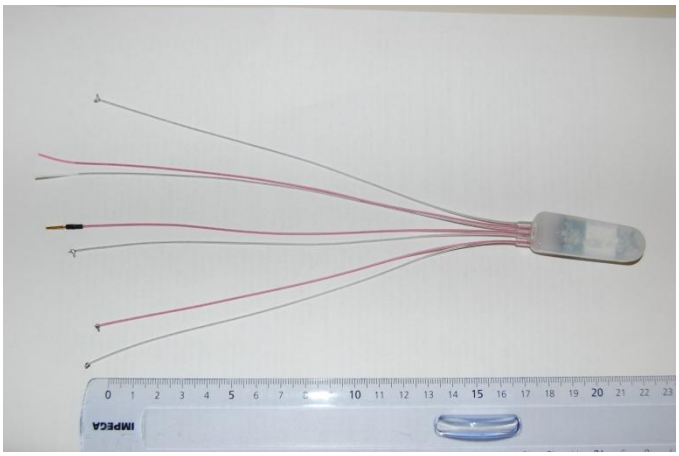


Figure 5: *The telemetric transducer type TL10M3-F50-EEE prepared for cortical EEG (channel 3), hippocampal EEG (channel 2) and EMG (channel 1) recordings.*

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-saggital 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 6). 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.

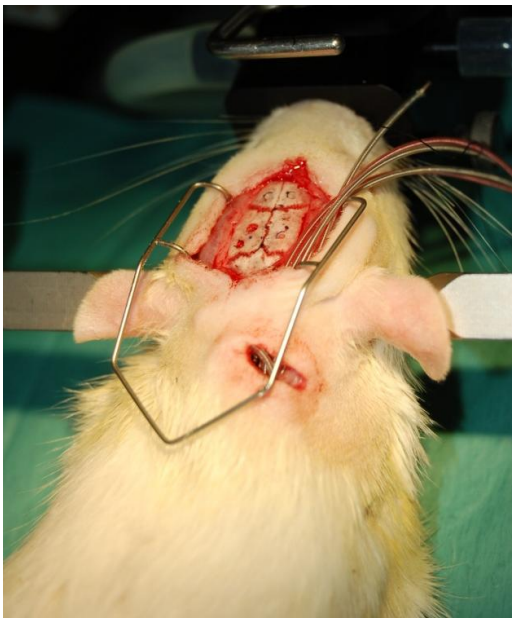


Figure 6: *Two incisions for placement of the EEGs leads on the scalp and EMG leads on the back of the neck for EMG.*

The two electrodes for EMG recording were secured to the underlying *musculus cervicoauricolaris* (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 sterotaxic apparatus. The skin on the scalp was reflected using a retractor to expose the entire dorsal portion of the skull. The periostium 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 (Figures 7- 8) and was initially hold in place with a small quantity of cyanoacrylate gel (RS Components, Northants, UK).

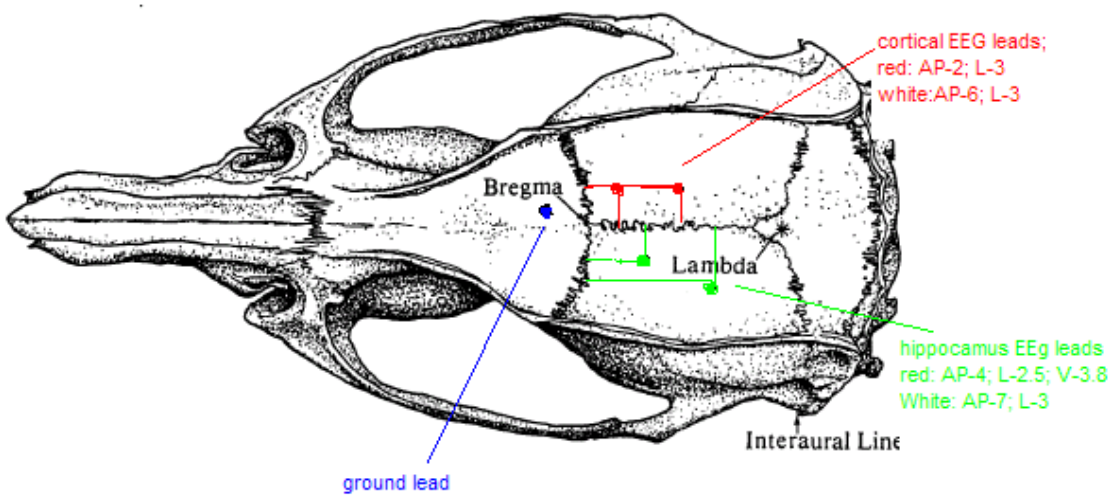


Figure 7: *A scheme of stereotaxic positioning of all the telemetric leads on the cranium.*

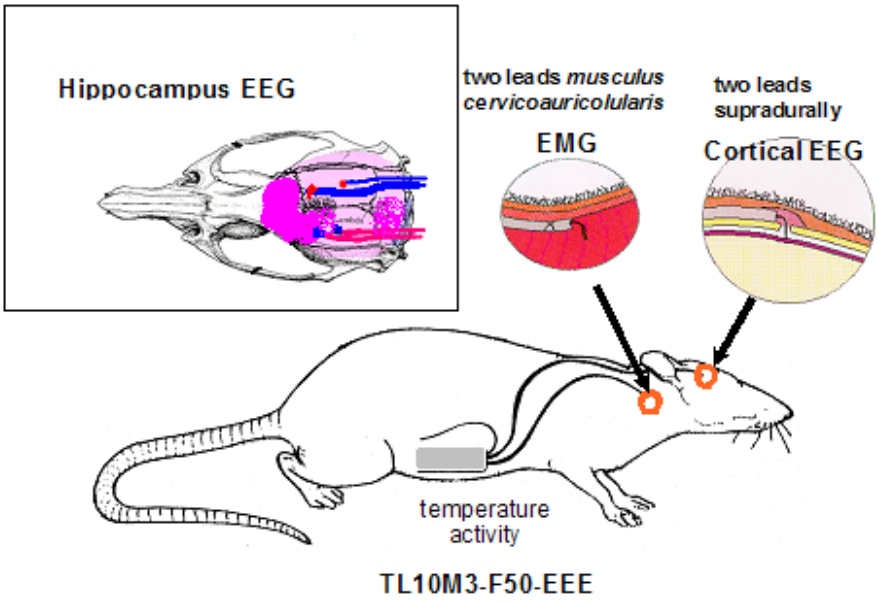


Figure 8: *A scheme of the surgical implantation of the telemetric transmitter in the rat.*

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 9-10-11).

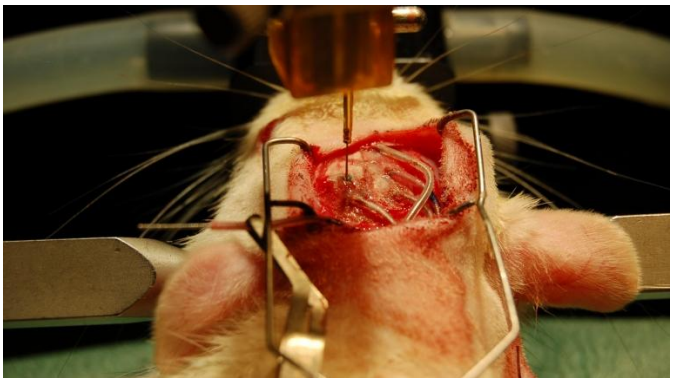


Figure 9: *The intra-hippocampus electrode attached to a guide wire is positioned deep in the left hippocampus.*

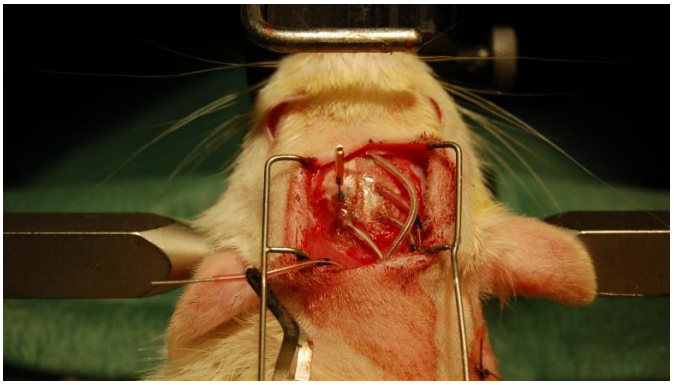


Figure 10: *The intra-hippocampus electrode is hold in place with a small quantity of cyanoacrylate gel.*

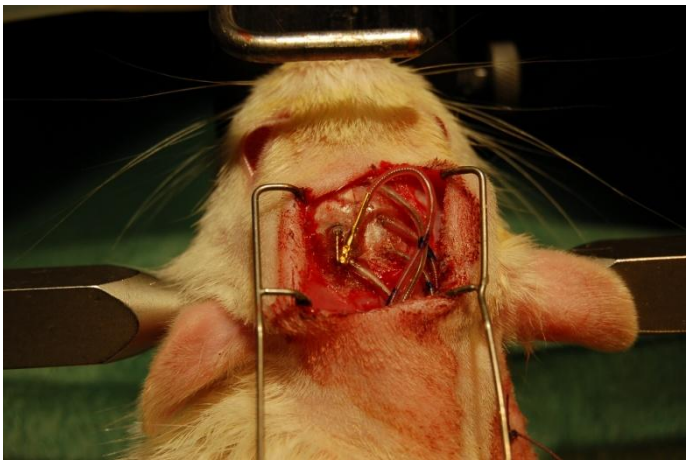


Figure 11: *The intra-hippocampus electrode is connected to the positive radio-telemetry lead (red) by mechanical contact. The negative lead (white) is placed supradurally caudal from the hippocampus lead.*

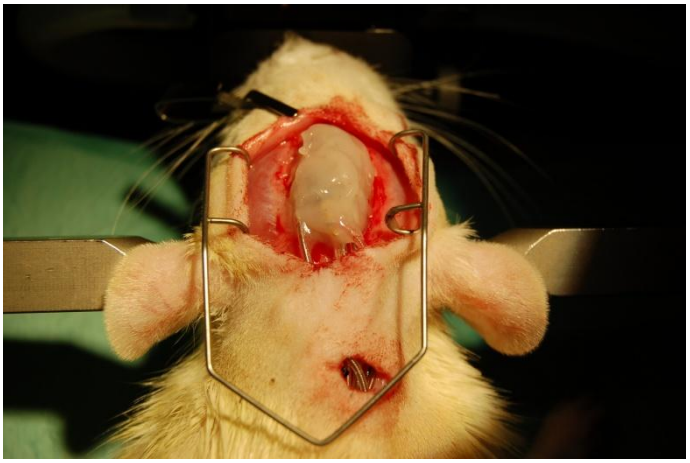


Figure 12**:** *All electrodes are covered and fixed to the skull with dental acrylic cement.*

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 12). 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.

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.

Post-mortem investigation

Under deep isoflurane anaesthesia a low current of 10mA for approximately 10-20 seconds were administered thought 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 13).

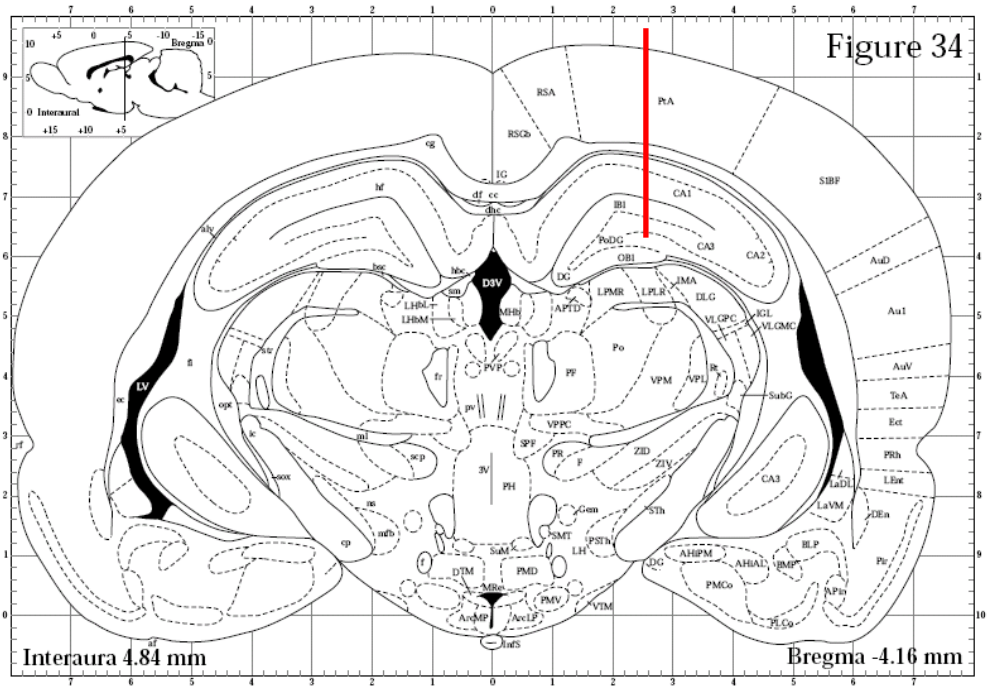


Figure 13: *The desired position of the deep electrode into the left hippocampus (dentate gyrus) based on stereotaxic coordinates of the Paxinos & Watson Atlas.*

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 (Figures 14-15).

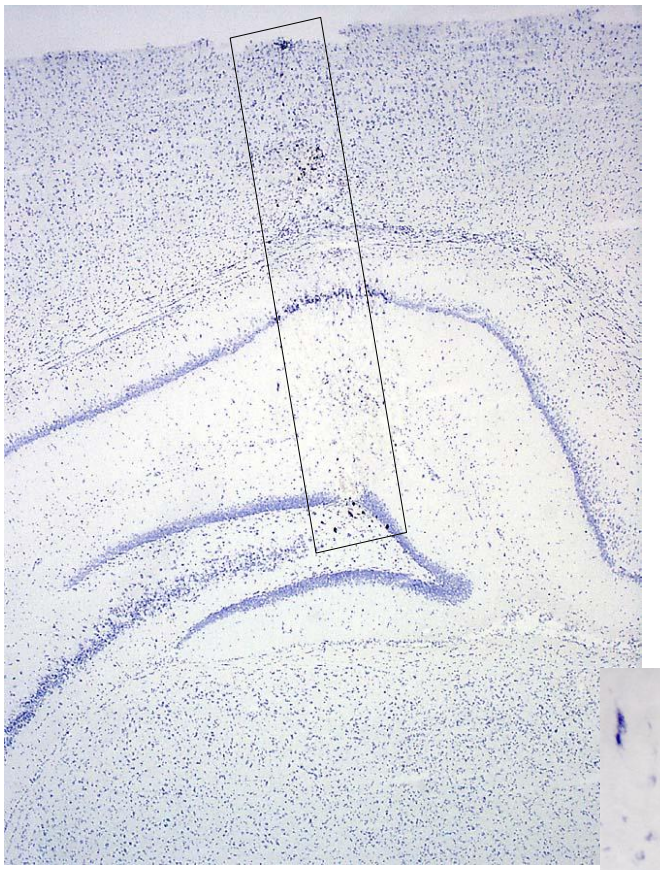


Figure 14: *The trace leaved by the deep electrode from the cortexdown to the neurons of the dentate gyrus of the left hippocampus (Nissl, 2.5X).*

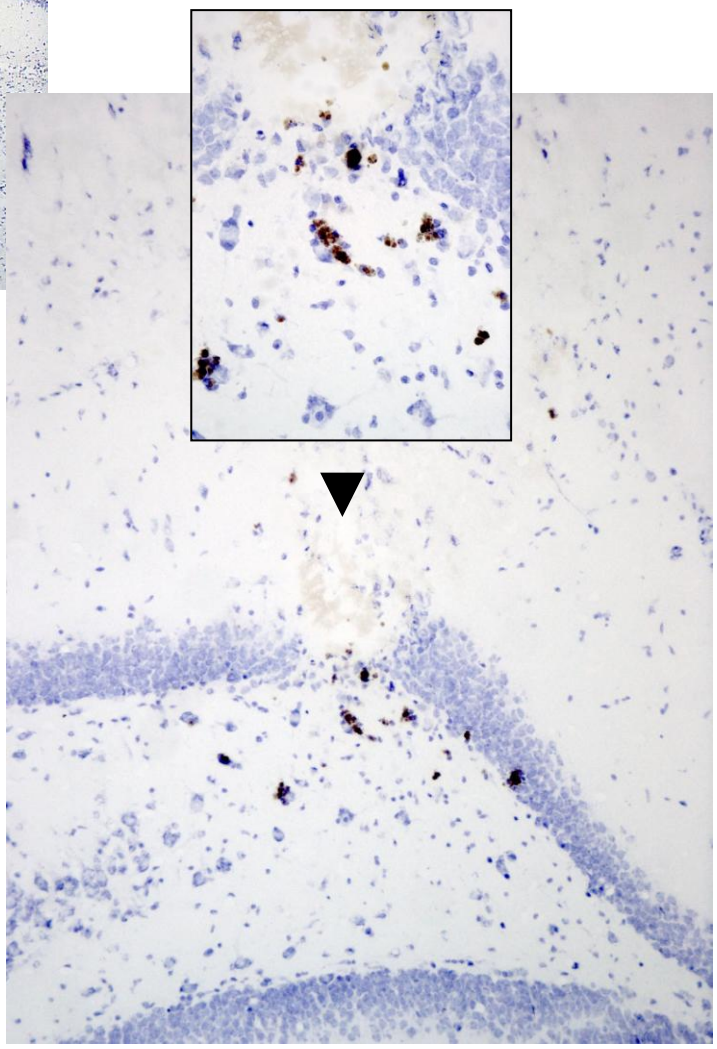


Figure 15: *A particular on the position of the electrode's active tip (Nissl, 10X and 40X).*

General considerations on this surgical technique

The developed surgical procedure for the implantation of DSI telemetric three-biopotential transmitters allows for a gradual recovery of animals within 15 days (Moscardo E and Rostello C, 2010), as also suggested in literature (Weiergrabrer M et al., 2005; Kramer K and Kinter LB 2003).

It was established that the behavioural and locomotor parameters, which are a good indicator of the health condition of the animals, showing a significant increase during the post-surgery period, indicated 15 days as the necessary period for recovery of behavioural changes, induced by the acute abdominal pain of the laparotomy (Roughan JV and Flecknell PA, 2001). Using a mechanical connection between the deep and the telemetric electrodes, the 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 hippocampal traces. In fact, in the hippocampus EEG, the Theta activity of neurons in this area of the brain was clearly identifiable.

The surgical technique of radio-transmitter implantation described above, which was developed and applied to rats to record simultaneous cortical and hippocampal EEGs, required a 15 day recovery period, both in terms of health status and quality of telemetric signals. Therefore, a recovery period of a minimum of 15 days is recommended before using implanted rats for experimental activities, in order to have good quality data and avoid interferences which could complicate data analysis and interpretation (Moscardo E and Rostello C, 2010).

The equipment set-up for video-telemetry EEG

A system capable to simultaneously assess the animal behaviour and the electrophysiological activities of the brain was set up in order to obtain an integrated acquisition and analysis of EEGs physiological traces and the behaviour in freely moving animals. A complete 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 16).

Figure 16: *The final Video-tEEG system installed in our laboratory.*

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.

The components of the installed Video-tEEG set-up (Figure 17) are:

- Video Acquisition system:

    - 2 Observer XT with External data module and multiple video module, to control 3 PhenoTyper® 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 (Panasonic WJ-MS424 Colour Quad System), each receives images from 3 camera top unit in order to perform on these images the automatic tracking of video data;

    - 6 lateral cameras b/w (by Ikegami black/white camera ICD-49E with Spacecom ottica varifocal manual iris 3-8 mm, F1.4, IR correct) in order to collect images in full-resolution allowing the detailed analysis of the behaviour.

- 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).

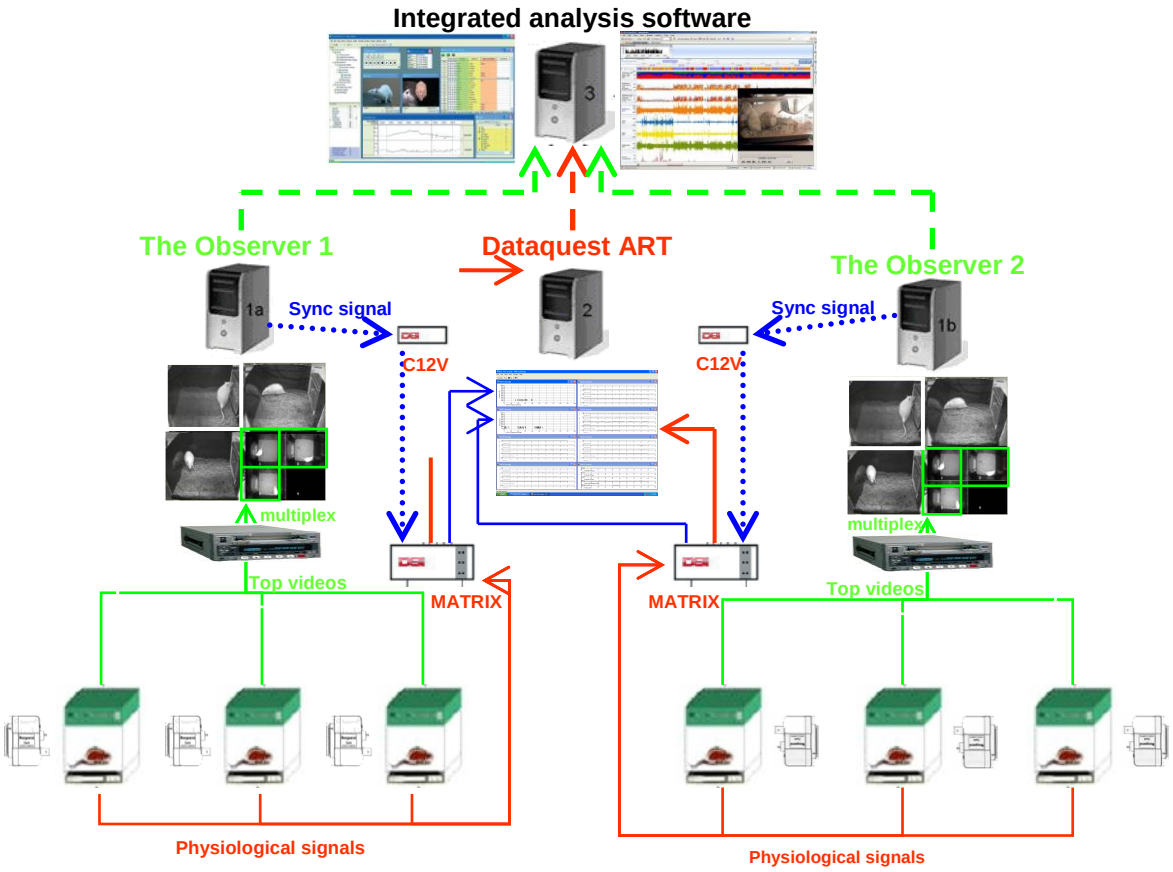


Figure 17: *The final set-up for video and telemetric data simultaneus recording.*

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 18). 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.

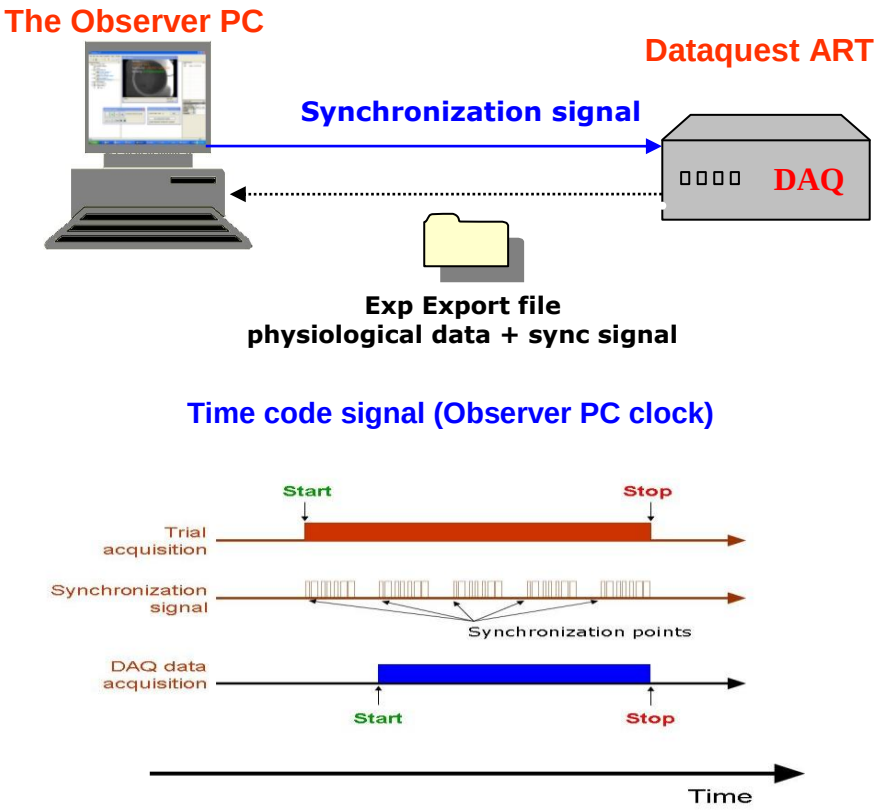


Figure 18: *How the synchronistation of video and telemetry sistems works.*

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 19). 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 distance moved, velocity, mobility, and movement thought all the video length (24 hours) (Figure 20). Then, analysis results of the automatic video tracking can be exported into Excel for following statistical analysis and graphs.

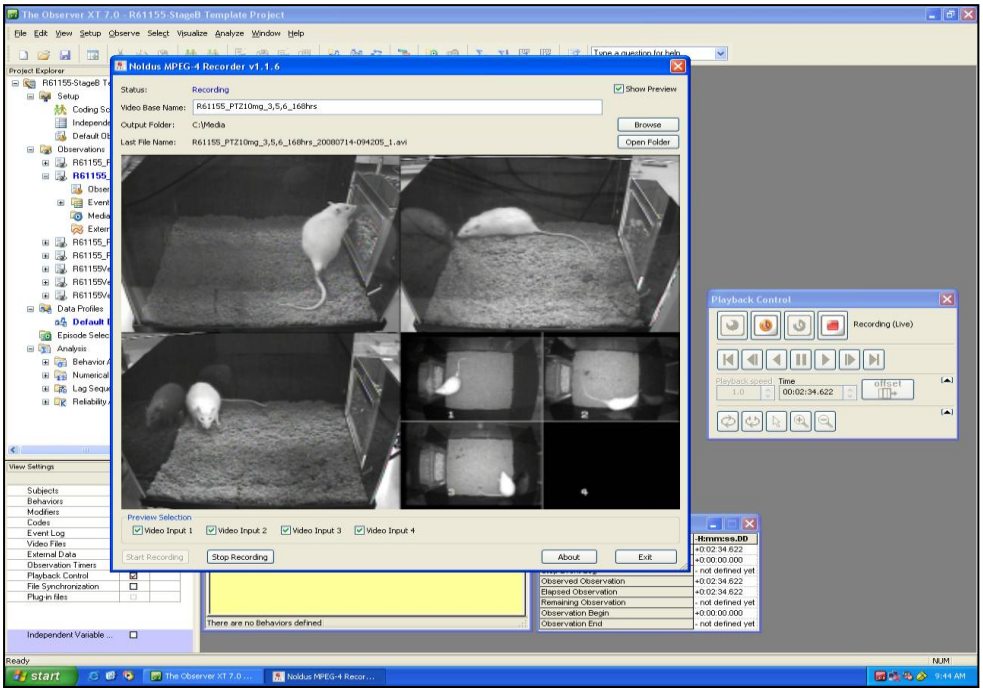


Figure 19: *The screen of The Observer PC during the acqusition of video signals. The first 3 images come from the lateral cameras, while the fouth image is the multiplexed results of signals from 3 Phenolab cameras top units.*

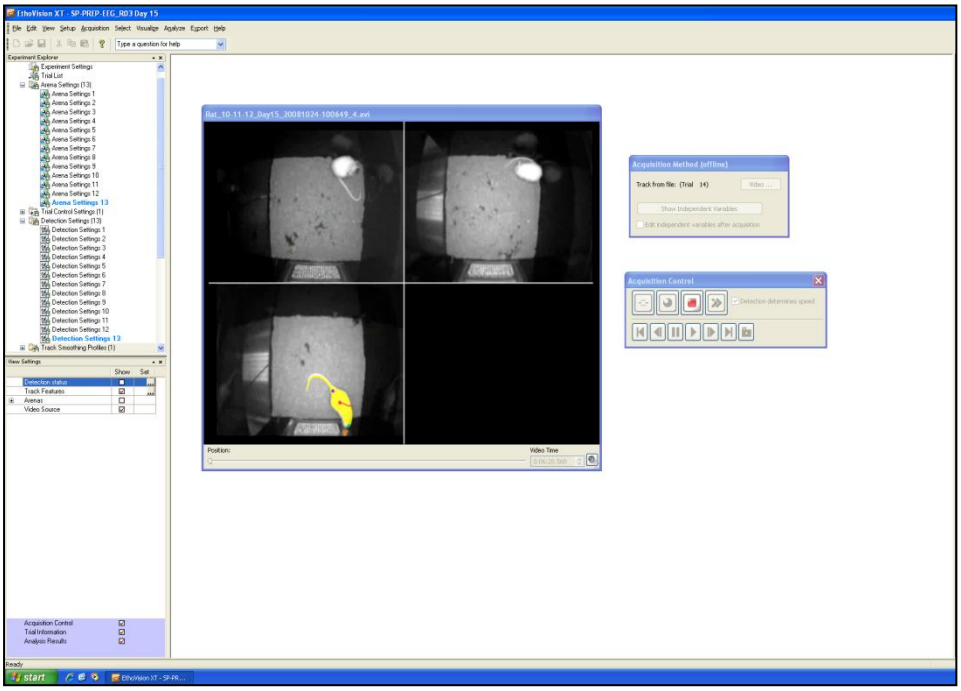


Figure 20: *The authomatic video tracking performed using EthoVision XT software on the multiplexed video signal which come from 3 Phenolab cameras top units.*

Video data can also be rearranged to be 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 21). 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.

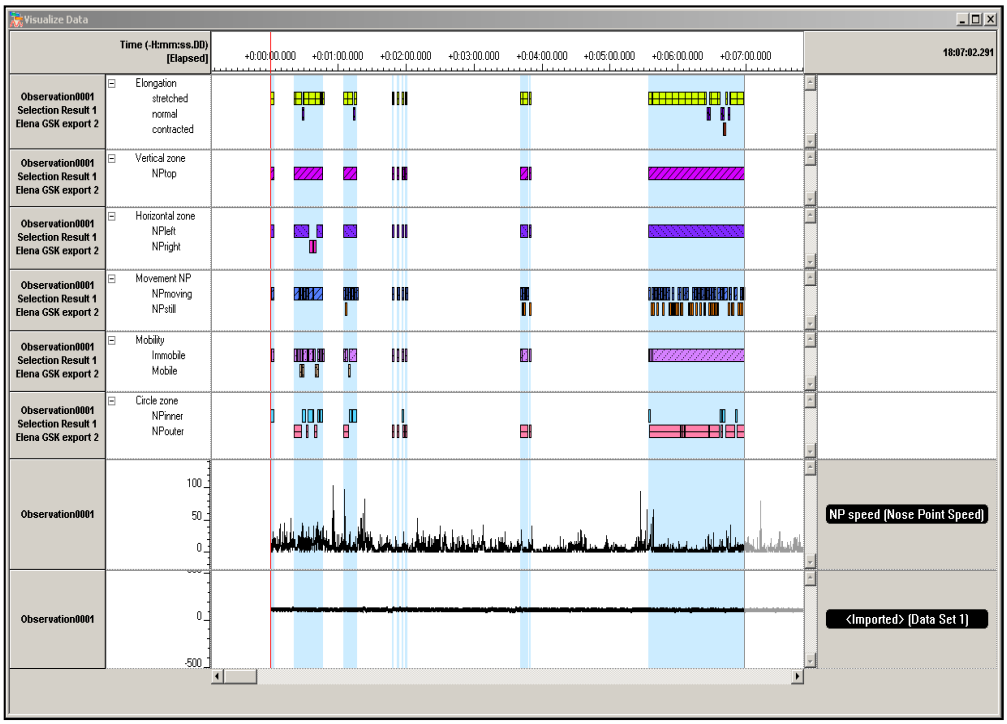


Figure 21: *Specific seleced episode/occurences together with synchronized physiological signals (EEGs traces) can be imported into The Observer XT for the manual detailed analysis.*

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 22).

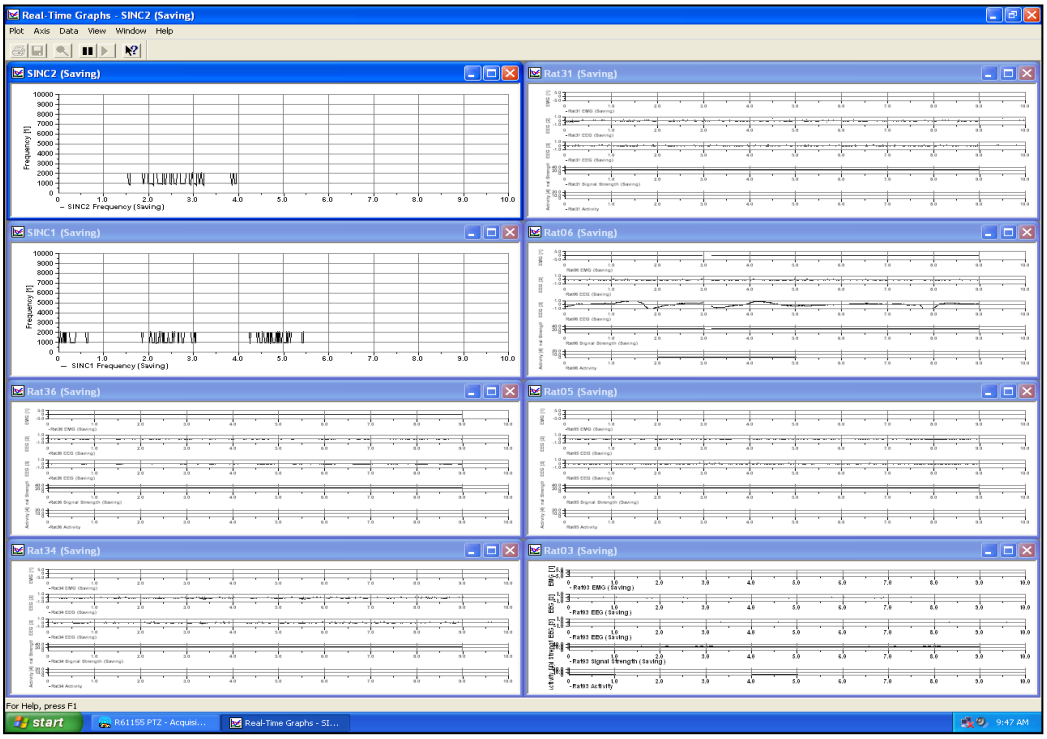


Figure 22: *The screen of ART Dataquest PC during the acqusition of telemetric signals. The first 2 are the synchronisers quad signls form the 2 Observer PC, the other 6 are the telemetric EEGs, EMG, Activity signals from 6 animals.*

In order to synchronize physiological data with video data, the EEG and EMG waveforms acquired with Dataquest ART have to be converted into txt. files and then imported into The Observer XT software as external physiological data together with synchronized video data. To export the DataQuest data is necessary to follow this procedure:

- Open the DSI Analysis module and from the File menu, select Load Data;
- In the Load Data window, in the Series tab, select Manual, clear the Continuous checkbox, put the Segment duration (for example 4h) and click OK;
- Select the files you want to export, press the right mouse button and choose Export Data and Into File (Figure 23).

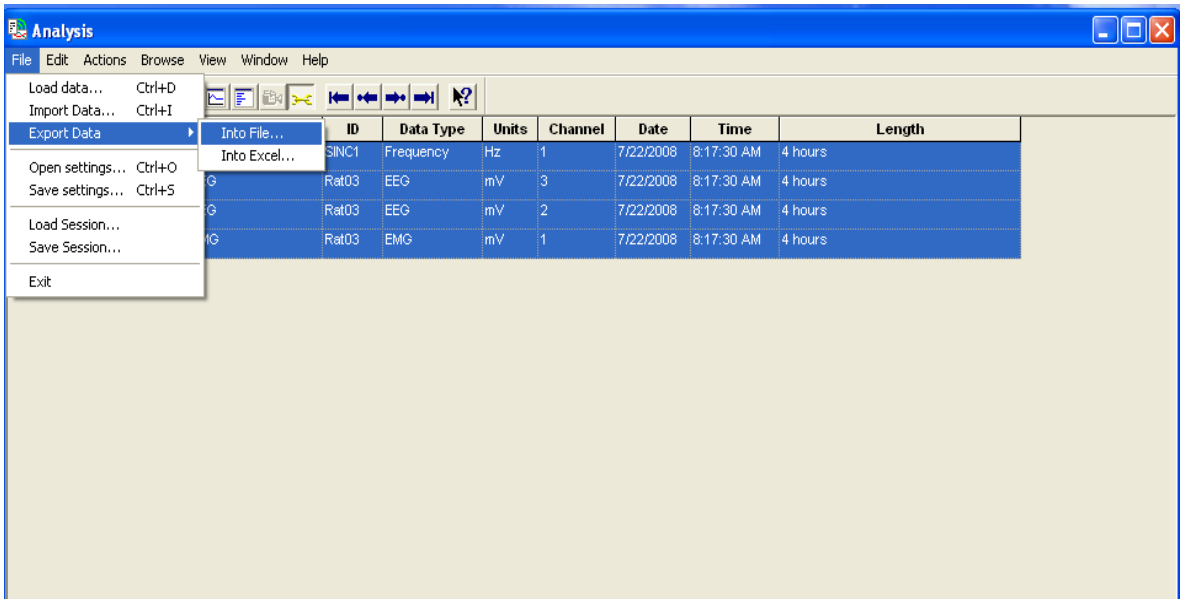


Figure 23: *The screen of ART Dataquest PC during the export of four DSI datafiles (in this example, two EEG data, one EMG data and one with the syncronization information from the Observer syncronization signal).*

- In the Export Setting window, select (Figure 24):
  - The Comma button in the Delimiter group
  - The File header checkbox
  - First segment in the Start at group and Count 1 in the Number of segments group to export the first segment period (for example 4h period);

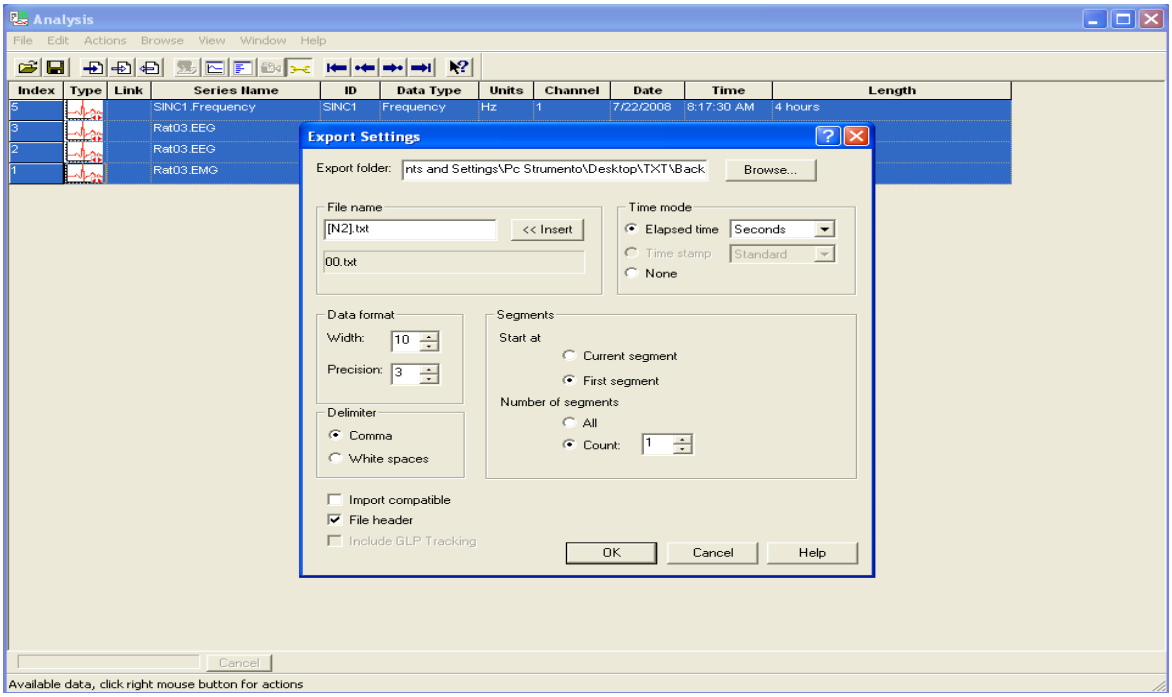


Figure 24: *The screen of Export Settings window in the DataQuest Analysis module.*

- Click OK to export the data to txt files.

After this export procedure is necessary to import the DSI DataQuest data into The Observer XT. To execute the import procedure is necessary to follow these steps:

- Open the Template Project and from the File menu, select Import and External Data;
- In the Import External Data window (File name) select the file .txt you want to import and the Files of type (Figure 25);

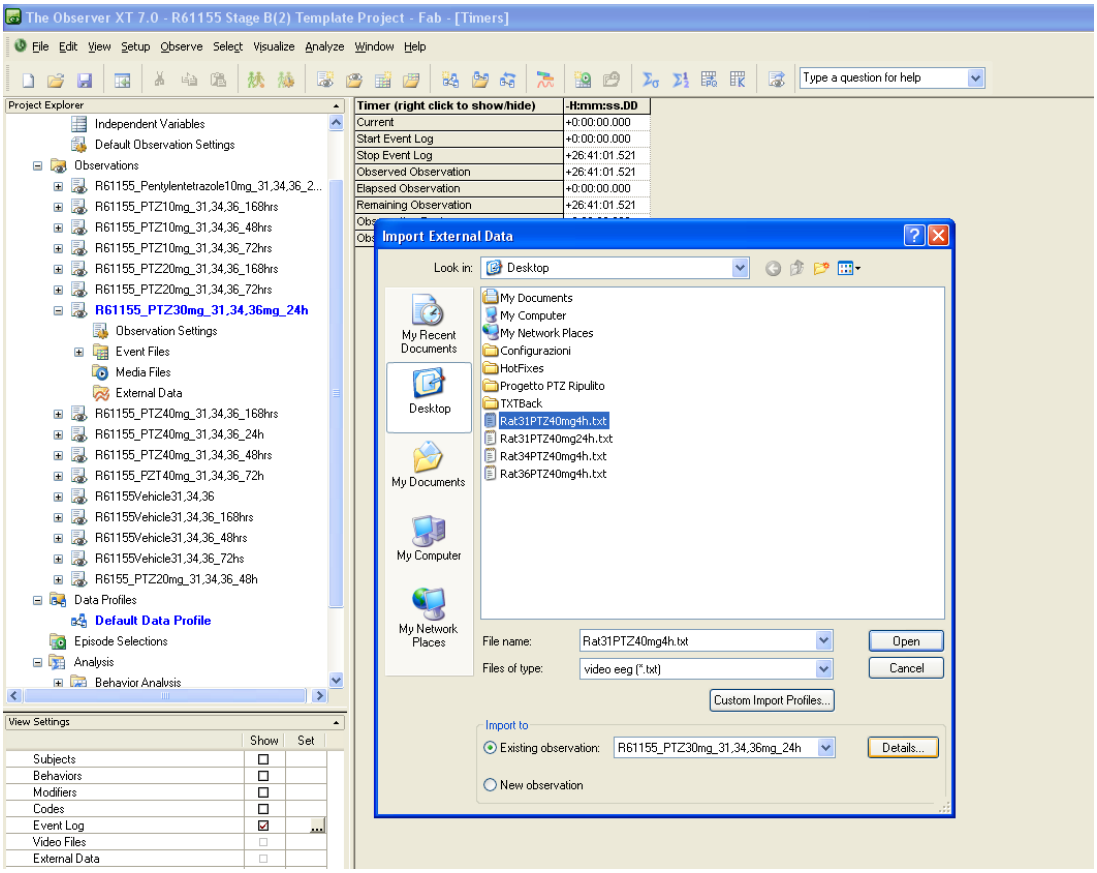


Figure 25: *The screen of Import phisiological data and synchonization signal in the Observer XT Project.*

- The Files of type have to be previously customized selecting Custom Import Profiles and filling in the data set based on experimental needs, as described in Figure 26;

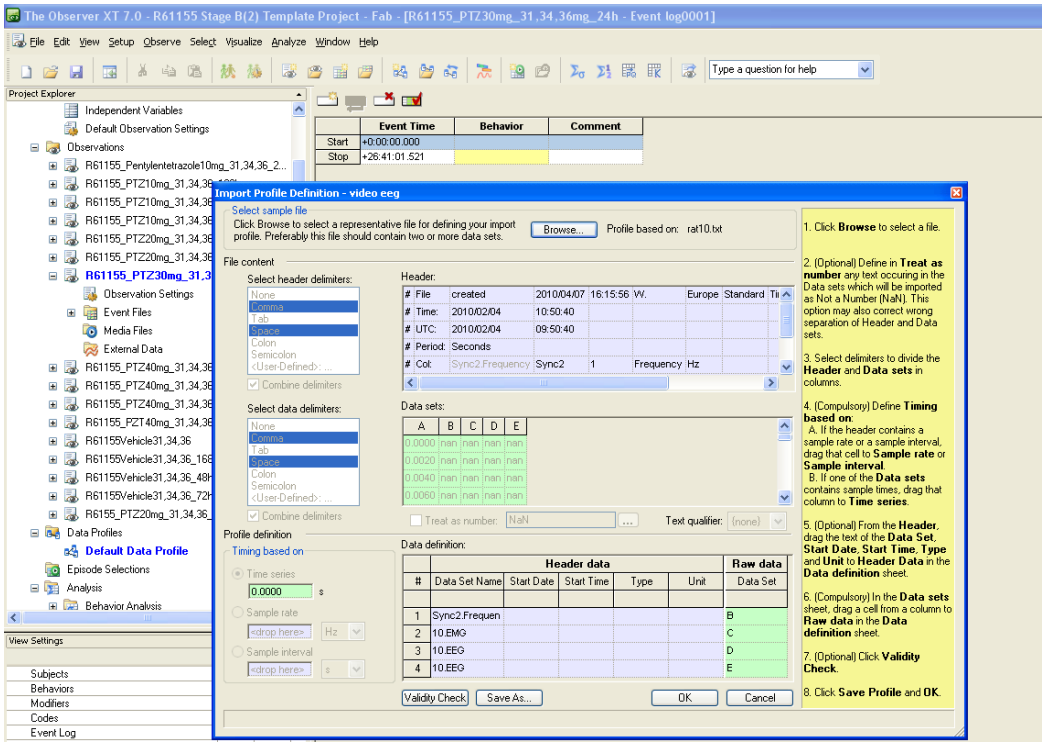


Figure 26: *The screen of window which describes the procedure to Import a profile of data set.*

- In the Import External Data window, select Details in order to open a sub-window, where it is possible to select and drug all the physiological data (i.e., EEGs, EMG) and the synchronization signal you are importing to the correct observation column (Figure 27);

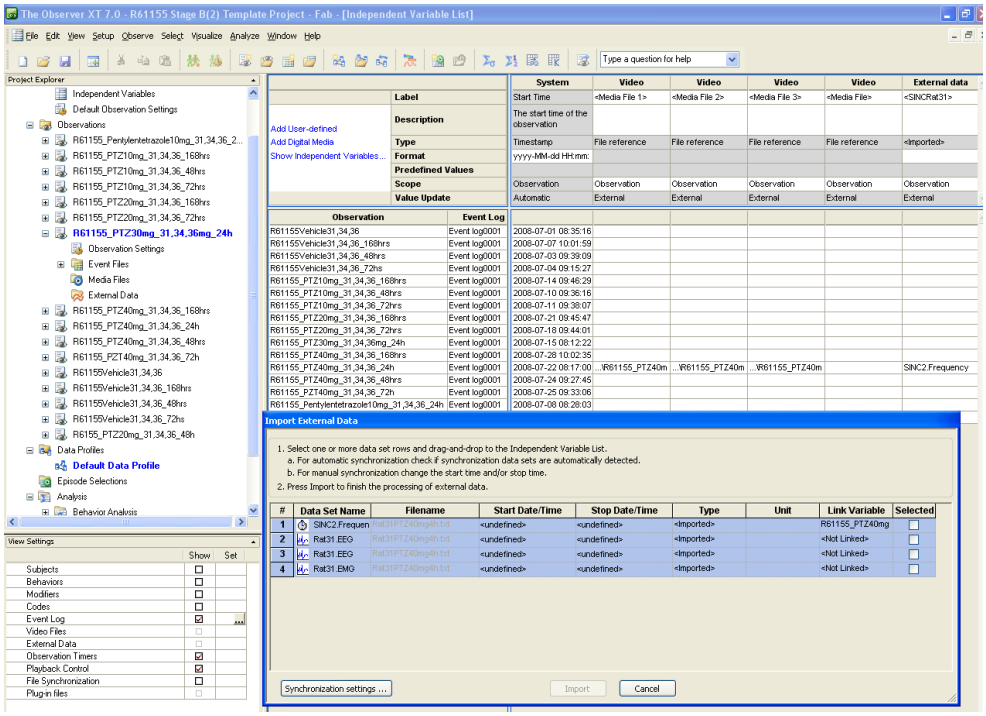


Figure 27: *The screen of details window which shows all data (EEGs, EMG and synchronization signal) to drag to the correct obsarvation column.*

- Click Import and then attend few seconds to finish the import data;
- Then will appear a final screen with both the video window and the Visualized Data window with the imported physiological data traces (Figure 28).

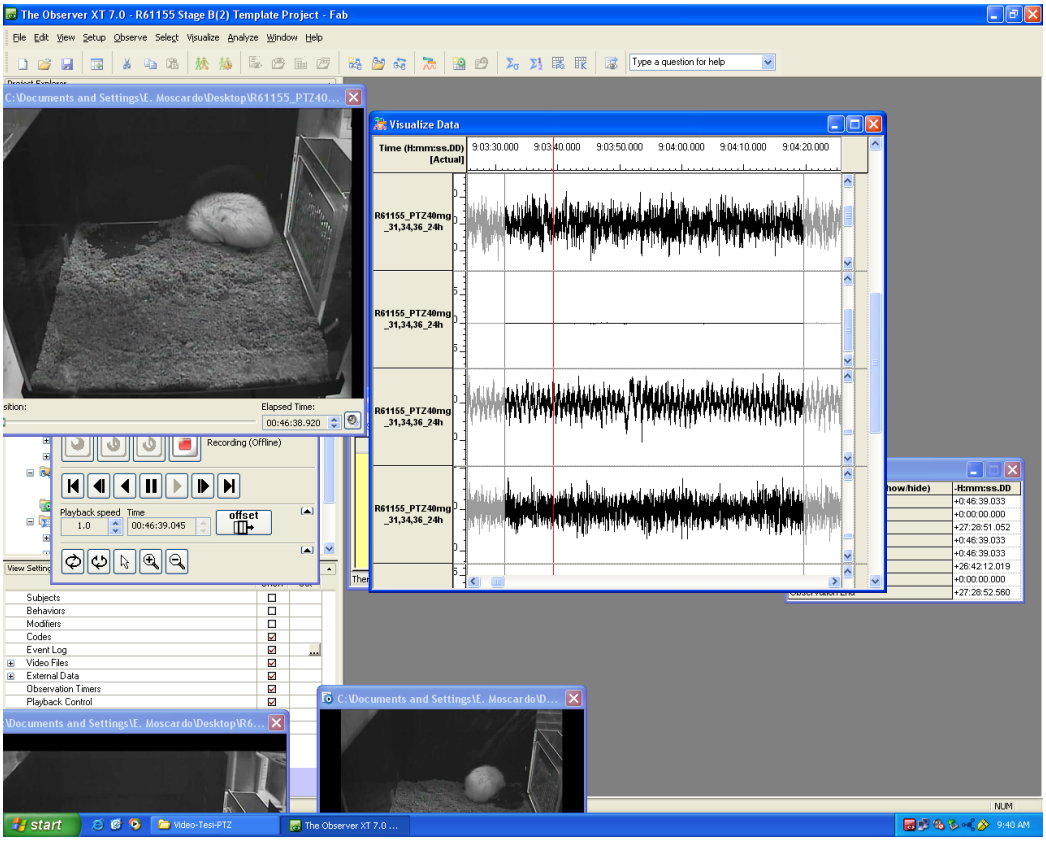


Figure 28: *The final screen after import of external data (EEGs, EMG and synchronization signal) to The Observer XT; video and phisiological data are shown in syncronized way.*

The EEGs and EMG physiological traces obtained by telemetry should be analysed having videos on the screen using dedicated software, because video information allows visual assessment of the subject's behaviour, confirmation of scoring events (Weak, Sleep, REM-Sleep), classification of convulsions severity levels, identification of seizure types and more.

Using the Neuroscore DSI software, videos and synchronised physiological traces can be shown at the same time on the screen (Figure 29), but a duplicate video acquisition (in addition to that obtained with The Observer) is necessary, therefore this option was excluded in our current Video-tEEG system set-up. The current analysis of EEGs and EMG physiological traces, visualised by Neuroscore software, is performed by a manual/visual review of all the traces, identifying the electrical alterations (i.e., seizures

and spikes trains) and marking the waveforms with the appropriate marker's label. Then, a check on the videos is performed to confirm the correct analysis and events identification.

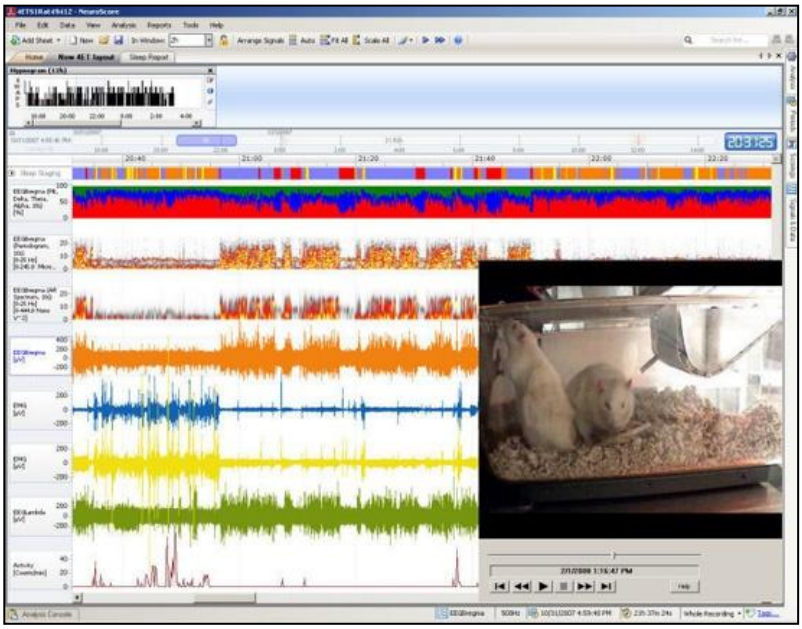


Figure 29: *The screen of Neuroscore DSI software as appears during the analysis of telemetric signals with the sysncronised video data.*

Advantages and Disadvantages of the Methodology

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 video-tEEG 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;
- 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;
- 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;
- the reduction of the inter-individual variability is allowed: the physiological parameters recorded continuously on the same individual animal also provide chronobiology information;
- the other pharmacological entities or electrical stimulation that can cause unwanted effects as secondary lesions resulting from abnormal activity of neurons or acute neurotoxic effects can be avoided.

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

- EEG has limited spatial/anatomical specificity, because electrodes pick up synchronization of neurons (amplitude of about 100 µV on the scalp and about 1-2 mV on the surface of the brain);
- DSI transmitters currently generate a maximum of three bio-potential channels (electrical activity of more than two-three discrete brain's areas cannot 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);
- it is labor intensive, technologically complex and expensive;
- large amount of video data (storage and archive considerations) are produced;
- the technological complexity of the EEG-video-telemetry system makes the GLP-validation difficult.

4. Experimental activities

Objectives and Rationale

The activities conducted during this research were associated with different applications of the Video-tEEG Model. The model was applied in two different conditions:

(PART I) Physiological conditions:

- *Establish relations between environmental condition, behavior and physiological parameters.*
  Analyze the relation between environmental condition (enrichment or not, olfactory stimuli or not) and behavioural/sleep pattern (EEG traces and/or behavioural tracking versus environmental condition) in rats with the objective to establish influence of different housing on physiological parameters.

(PART II) Pathological conditions:

- (a) *Relation between seizures and convulsions.*
  Analyze the relation between seizures and convulsions (EEGs traces versus behavioural observations) in rats with the objective to understand the power of the EEG-telemetry Model for assessing the seizure-convulsive potential risk of New Chemical Entities (NCEs). In particular the aim of this part is to characterize and quantify the non-convulsive electrical activity and differentiate them from the convulsive seizures induced by PTZ.

- (b) *Evaluate the feasibility of combining the Video-tEEG model with a PK-PD modeling.*
  Assess the technical feasibility for the integration of a Video-telemetry Electroencephalography (video-tEEG) system with a set up for sequential blood bleeding in the laboratory rat. This combined model is considered potentially extremely helpful for a reliable pharmacokinetic/pharmacodynamic (PK/PD) modeling of behavioural and EEG changes following the administration of a new candidate drug by a time-right identification of plasma concentrations.

Statement of ethical approval

All the experiments described in this research work were carried out in accordance with Italian regulations governing animal welfare and protection and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and according to internal GlaxoSmithKline Committee on Animal Research & Ethics (CARE) review.

Materials and Methods

Animals and Housing

Thirty-four male Sprague-Dawley CD^{TM} rats (Crl:CD (SD) IGS BR) supplied by Charles River Italia, Calco (LC), Italy were used for surgical implantation of telemetric devices (as described into section 'Transducer probe surgical implantation, pag 13). 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.

Free access to standard food pellets (Altromin R®, Rieper, Germany) and filtered water 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.

PART I:

PHYSIOLOGICAL CONDITIONS

Establish relations between environmental condition, behavior and physiological parameters

Materials and Methods

Eight animals surgical prepared as described into section “Transducer probe surgical implantation” (pag. 13) were selected for this first objective on the basis of their clinical conditions, the quality of telemetric signals and general behaviour.

Forty-five days by surgery day, 8 animals were put into experimental PhenoTyper® cages. After 6 days of habituation, 4 of 8 animals received novel environmental enrichment (3 objects: a tunnel, a ball and a mouse house) (Figure 30). EMG, cortical EEG (EEG_c) and hippocampal EEG (EEG_{hipp}) signals by telemetry and the general activity data by video were recorded for 24h immediately after this enrichment change (Phase 1) and after 3 days of permanence of the animal into the enriched cage (Phase 2).

Figure 30: *Experimental cage (PhenoTyper® Cage) with environmental enrichment.*

Three days after this last registration, all animals received olfactory stimuli. Two drops of bergamot and 2 of lemon essential oil (Specchiasol srl, Verona, Italy) applied randomly into bedding every morning (7.30-8.30 a.m.) for 4 days. From these rats 24h telemetric signals (EEGc, EEGhipp and EMG) and videos were recorded immediately after the first olfactory stimuli (Phase 3) and on the 4th day of olfactory stimulation (Phase 4). Telemetric and video recordings were made with the animals singly housed in their

PhenoTyper® cages and all the Video-tEEG system's equipment previously described (section 'The equipment set-up for video-telemetry EEG, page 22) was used (Figure 31).

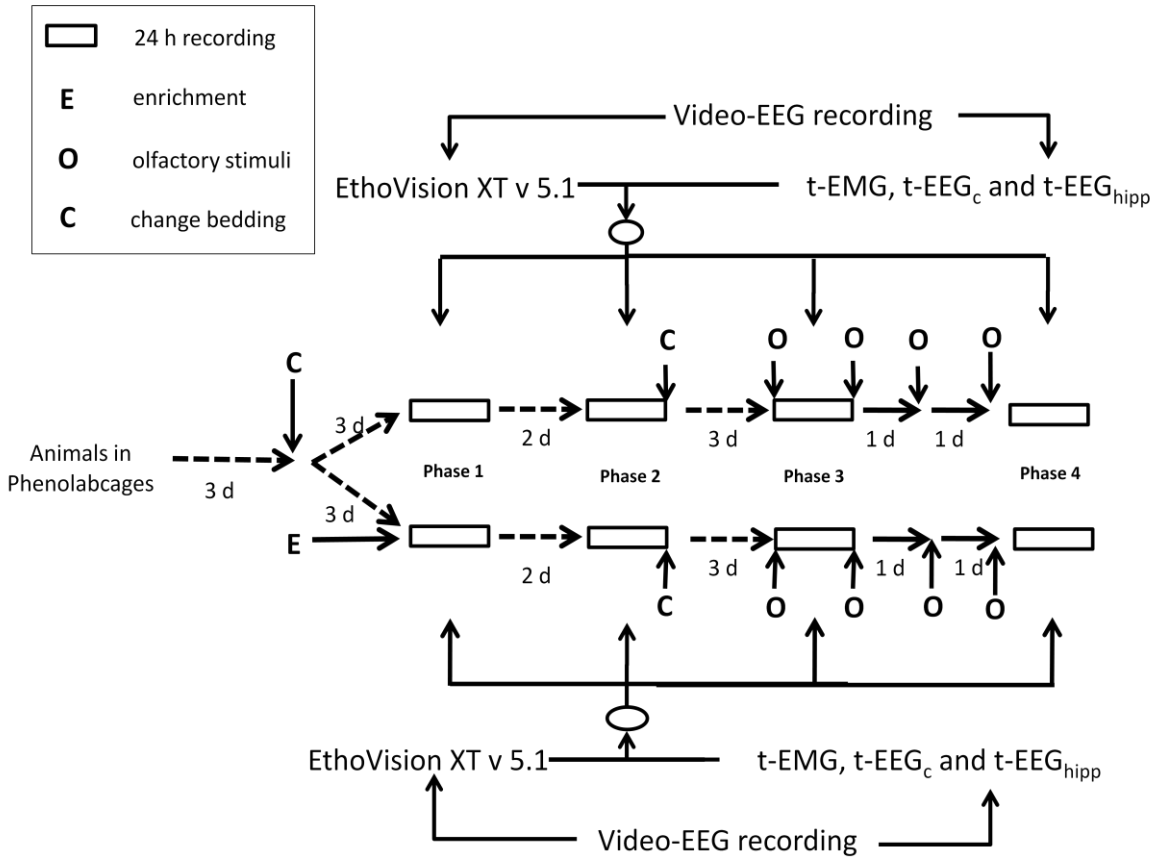


Figure 31: *Schematic representation of the experimental plan.*

Recording and analysis of videos and telemetric signals

On 8 implanted animals, EMG, EEG$_c$ and EEG$_{hipp}$ signals by telemetry and the general activity data by video were recorded in each occasion.

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 8 rats and for the whole 24 hours period, the following parameters were evaluated in each occasion:

a) movement (the duration of moving of the center-body point);
b) mobility (the duration of mobile animal);
c) velocity (velocity of the animal's center-body point).

Telemetric data were collected using the hardware-software acquisition system *Dataquest ART Gold 4.01* (Data Science International). Telemetric EEGs and EMG traces of 8 implanted rats were analyzed for the whole 24 hours period using the custom Matlab scripts software in order to visualize the traces. A sleep analysis was performed manually in epoch of 10 seconds. Three stages were identified: wakefulness (W), synchronized wave sleep (SWS) and rapid eye movement (REM) sleep. The identification of each stage was performed when at least of 50% of the epoch met following criteria:

- *Wakefulness (W):* EEG_c appeared desynchronized with high frequency and low amplitude accompanied by elevated EMG activity;
- *Synchronized wave sleep (SWS):* EEG_c appeared synchronized with low frequency and high amplitude accompanied by lower EMG activity;
- *Rapid eye movement sleep (REM):* EEG_c appeared characterized by theta activity (5-10 Hz) accompanied by isotonic EMG activity.

Theta activity (5-10 Hz) was instead manually analyzed during wake phase in all collected EEG_{hipp} traces (Figure 32).

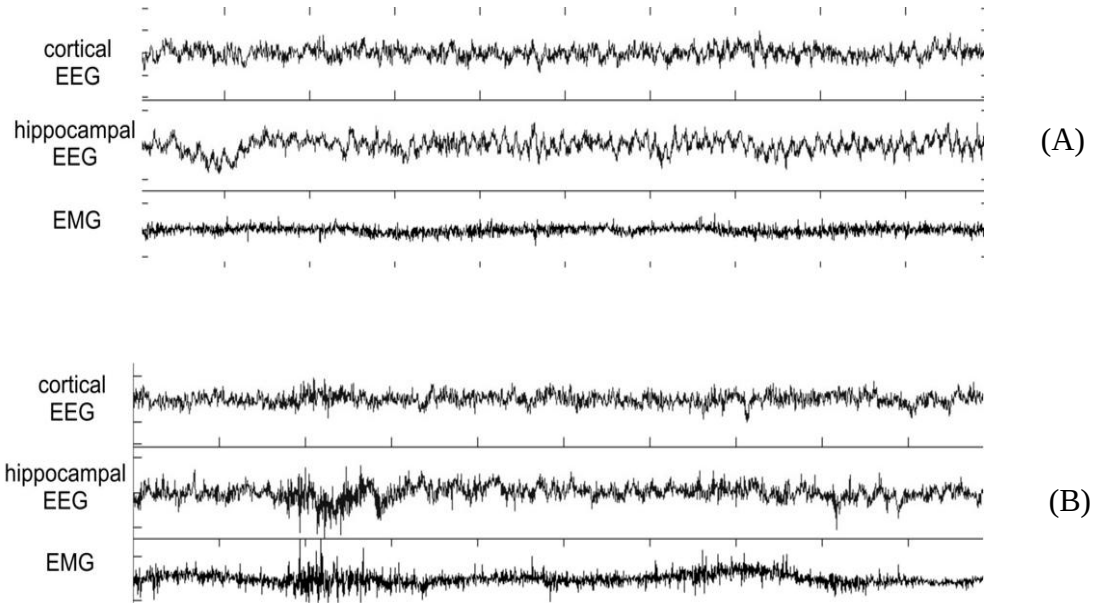


Figure 32: *Example of theta activity identification during wakefulness phase (W).*
(A) W phase with theta activity; (B) W phase without theta activity.

Statistical analysis

Statistical analysis of motor parameters and sleep data was performed using GB-Stat v 7.0 software (Dynamic Microsystems Inc., Silver Spring, MD, USA). One-way repeated measures analysis of variance (ANOVA) was used in the data analysis across days, using the Dunnett post-hoc procedure. No statistical analysis was used in theta activity evaluation.

Results

Analysis of the videos

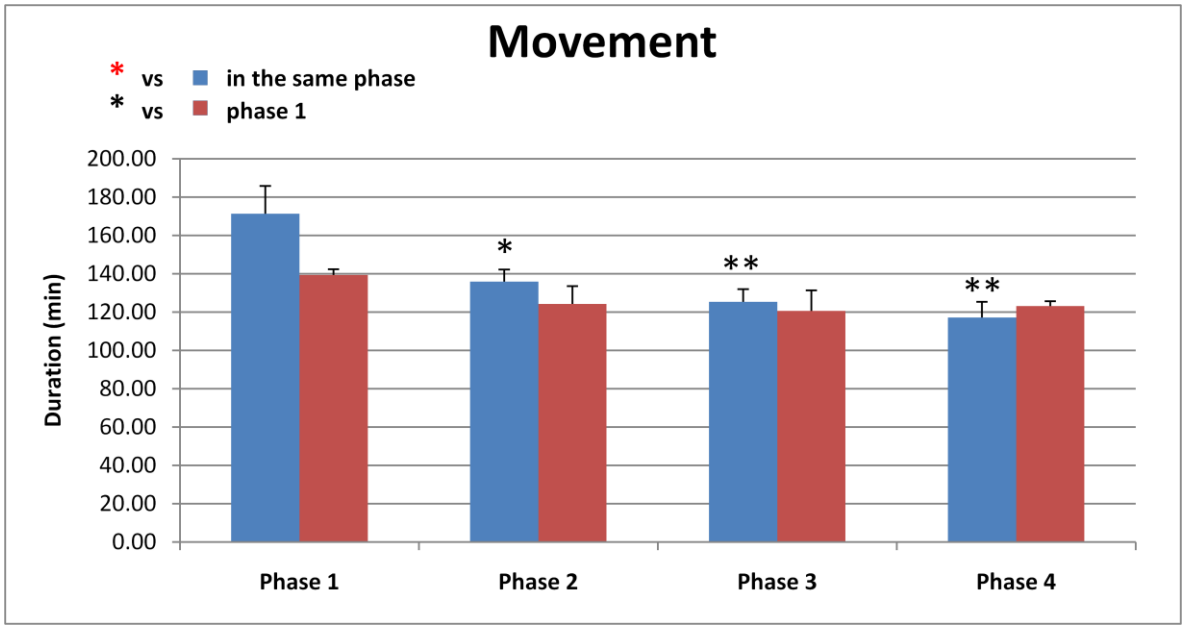
Three video files (1 of novel enrichment day -phase 1- in the not enriched environment group; 2 of novel olfactory day –phase 3- 1 in not enriched environment group and 1 in enriched environment group) were corrupted and it was not possible to analyze them using the automatic video-tracking software *Ethovision XT*.

No differences have been found in not enriched group in any motor considered parameters between different phases.

In Phase 1 of experiment, animals which received the novel environmental enrichment (NEE) were found to significantly be affected regarding mobility duration ($p<.05$ vs not enriched environment group), whereas movement duration and velocity fail to reach significant increase. Animals belonging to NEE showed an increased motor activity, when compared to the next phases (movement: $p<.05$ phase 1 vs phase 2, $p<.01$ phase 1 vs phase 3 and 4; velocity: $p<.05$ phase 1 vs phase 2 and 3, $p<.01$ phase 1 vs phase 4; mobility duration: $p<.05$ phase 1 vs phase 2, $p<.01$ phase 1 vs phase 3 and 4- see Figures 33-34).

No significant differences were observed 2 days after the introduction of the environmental enrichment (Phase 2) between enriched and not enriched of the same phase in any considered motor parameters.

After introduction of novel olfactory stimuli (Phase 3) and after 4 daily reinforced olfactory stimuli (Phase 4), no differences were found between enriched and not enriched environmental groups of the same phase in any considered motor parameters.



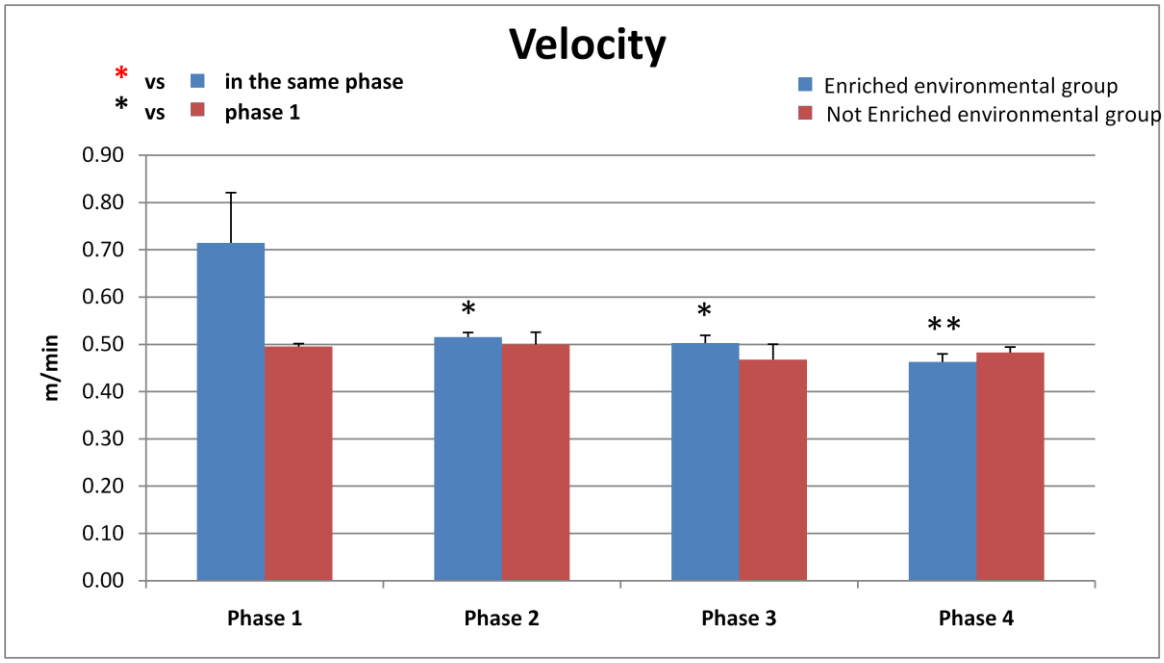


Figure 33: *Movement and velocity parameters analysed in different experimental conditions in 24h period of video recording (** p<.05, **p<.01*); Data are expressed as mean±SEM.*

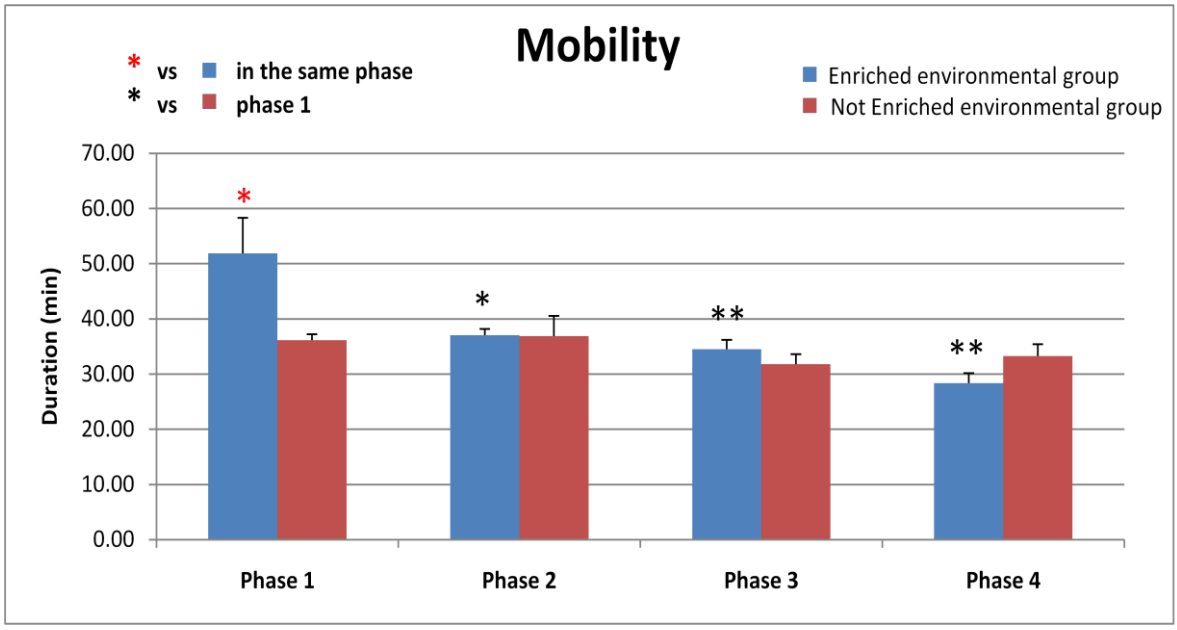


Figure 34: *Mobility duration parameter analysed in different experimental conditions in 24h period of video recording (** p<.05, **p<.01); *Data are expressed as mean±SEM.*

Analysis of telemetric signals

In each different phase of experiment, there were not differences in time spent on different sleep stages (W, SWS and REM). In all animals the time spent in wakefulness is higher during dark phase than light phase when rodents are in activity on the other hand SWS and REM are higher during light phase than dark phase (Figure 35).

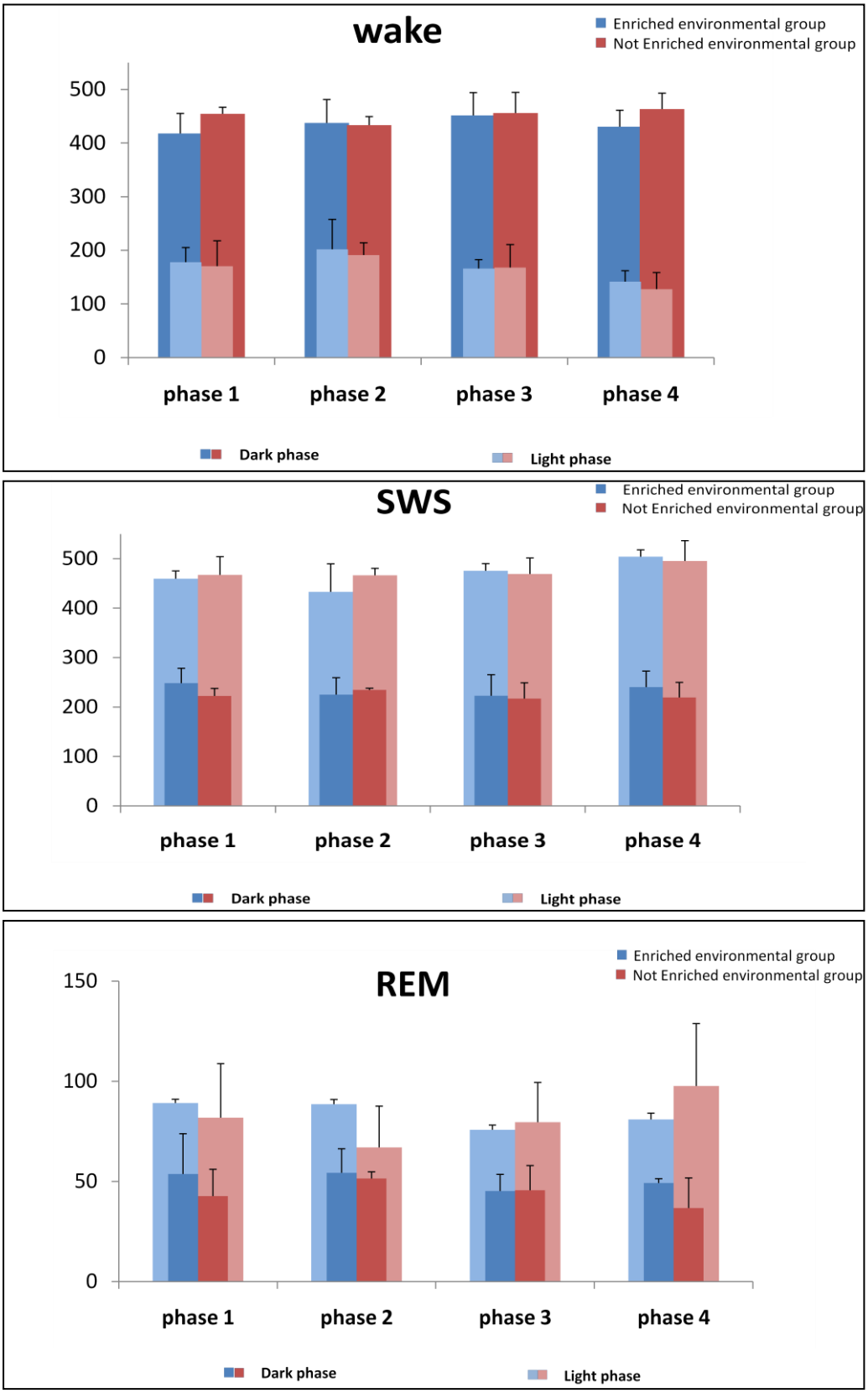


Figure 35: *Total duration (msec) of sleep stages (W, SWS and REM) in the different experimental phases.*

Regarding theta analysis, if it is considered the differences between the novel olfactory phase (Phase 3) and the novel enrichment phase (Phase 1) the total time of theta activity is a positive delta in the animals with environmental enrichment while is a negative in animals without enrichment stimuli. In both cases, the difference is below significant values. Considering the differences between olfactory phase (Phase 4) and enrichment phase (Phase 2), the delta is positive for all animals but the total time of theta activity is almost three times more in animals without than ones with enrichment stimuli (Figure 36).

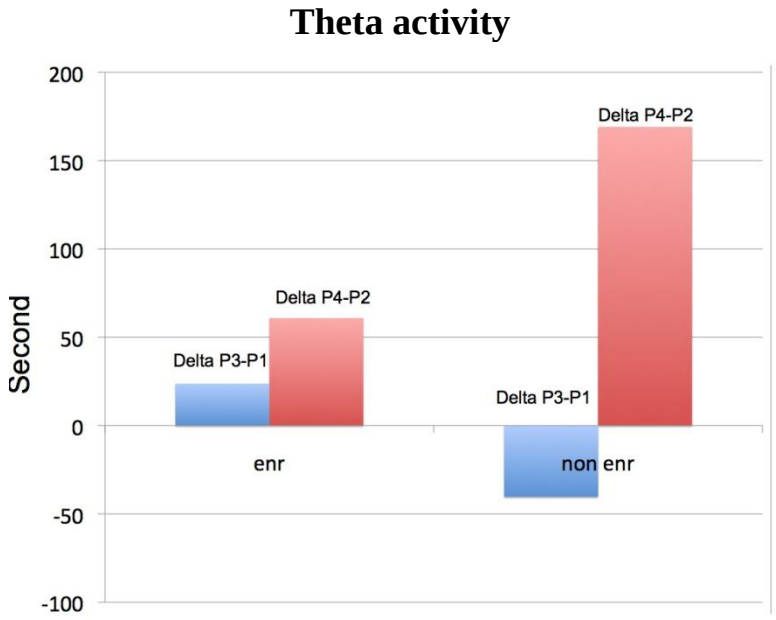


Figure 36: *The delta theta activity of different phases of experiment in the enriched (enr) and not enriched environmental groups (non enr)- (P3-P1=Difference between novel olfactory phase and novel environmental enrichment phase; P4-P2=Difference between olfactory phase and environmental enrichment phase).*

Discussion

The spontaneous behaviour analysis showed that the novel environmental enrichment induces an increase of motor activity which is observed acutely but not after 2 days of novel objects introduction. The novel enrichment enhances cage exploration resulting in an increase of the rat motor activity; after few days, probably due to animals adjusting to the enrichment presence, this increment disappears. Our data are in agreement with previous works (see, i.a., Townsend P., 1997 and Orok-Edem E., 1994), which reported

an increase in exploratory in animals housed in enriched cages. In contrast to these results, Abou-Ismail (2010) observed an analogue increase in rats in unenriched cages than ones in enriched cages; instead Foulkes A. (2004) found no differences in rats housed in conventional or enriched cages. These opposite findings could be due to the different experimental conditions such as period of habituation and/or observation. Furthermore, these ambiguous results could be explained with the presence of hidden variables, which are part of the major aims of this research.

Instead, the introduction of olfactory stimuli, induced by essential oil present in common detergent of laboratory tables, did not cause any changes in the mobility parameters. In general both animals in enriched and not enriched environment cages didn't show any differences in movement, velocity and mobility duration in novelty olfactory stimuli phase and after 4 daily reinforced olfactory stimuli.

The sleep analysis of this study didn't show differences in time spent on sleep stages (W, SWS and REM) in any different phases of experiment. Very few studies have investigated effects of enriched conditions on sleep pattern and no one of olfactory stimuli. These studies reported an increase in the REM sleep duration in animals housed in enriched conditions. These opposite results could be due to different experimental conditions above regarding the time spent in the enriched housing.

The theta activity analysis showed that there is a light difference between time of theta activity during the first day by introduction of enrichment or olfactory stimuli in all animals with or without environmental enrichment. Otherwise the interesting result is the value of delta considering the differences between olfactory phase (Phase 4) and enrichment phase (Phase 2) and so focus on the stimuli after "habituation". In details, the animals which didn't receive enrichment showed an increase of theta activity almost three times than animals with environmental enrichment. This result should indicate that a single stimulus (olfactory stimuli) induces an increase of theta activity bigger than more stimuli associated together. In literature it has been suggested that theta activity in the hippocampus and neighboring cortical regions may be involved in mnemonic processing (Chrobak JJ and Buzsaki G, 1998; Shen J et al., 1997) and the theta rhythm could be represented a "tag" for short-term memory processing in the hippocampus (Vertes RP, 2005). Our results could suggest that "multimodal" memory does not work as a mere summary of different memories, but instead that the brain store differently information depending on the modal nature (environment, smell, vision, etc).

Conclusion

The spontaneous behaviour analysis suggests how it is important to check any possible influence of test conditions and also the routine laboratory procedures like cleaning, in order to ensure the reliability and daily repeatability of experimental data.

Overall, the present findings seem to indicate that olfactory stimulations can have little, if any, effects when associated to other stimuli, such as novel objects or enriched environment; on the other hand, the effect will be dramatic in case of non-enriched housing. This effect can be explained either in terms of salience (the “stronger” stimulus wins) or in terms of sensory filtering. This latter point should be investigated further by the mean of new experiments.

PART II:
PATHOLOGICAL CONDITIONS

a) Relation between seizures and convulsions

Materials and Methods

Six Sprague-Dawley CD^{TM} male rats (Crl:CD (SD) IGS BR - supplied by Charles River Italy, Como, Italy) of approximately 7 to 8 weeks age, were surgical prepared as described into section 'Transducer probe surgical implantation (pag. 13), were selected for second objective of this research work on the basis of their clinical conditions, the quality of telemetric signals and their general behaviour.

All animals were injected with vehicle (0.9% w/v Sodium Cloride) and, the following day, with PTZ (Sigma-Aldrich, Milano, Italy) freshly dissolved in the vehicle and administered intraperitoneally at 40 mg/kg at a dose volume of 10 mL/Kg (for the schematic representation of the experimental plan, see Figure 37).

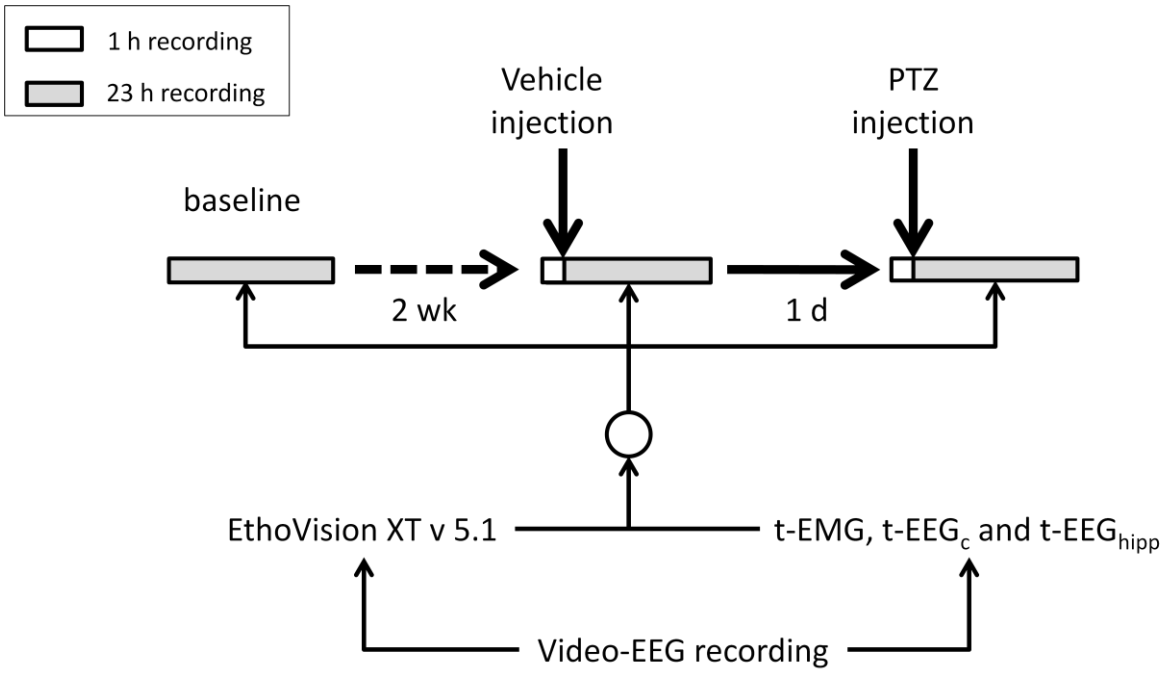


Figure 37: *Schematic representation of experimental plan.*

The dose was selected to provide recoverable generalized clonic or tonic-clonic convulsive seizures with a motor-behavioural component. This dose level was selected based on previous investigative studies in which PTZ was administered intraperitoneally to male Sprague Dawley CD^{TM} rats of approximately 12 weeks, at doses 100, 80, 50, 30, 25, 10 and 5 mg/kg. The dose of 100 mg/kg induced clonic or tonic-clonic 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. Taken into account that the susceptibility to PTZ-induced seizures is age-dependent (Klioueva IA et al., 2001) and the telemetered implanted CD^{TM} rats involved in this study were 7-9 months old, the dose of 40 mg/kg PTZ was considered appropriate for the study.

Clinical observations and Body weights

Animals were subjected to clinical check at least once daily. Clinical observations were performed before dosing (BD), immediately after dosing (IAD) and continuously for the four hours post-dose.

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

Telemetric measurements and video recordings

During treatment day, telemetric signals and videos were recorded simultaneously and continuously. An hour before treatment and 4 hours after treatment (data acquisition started at approximately 8:30 am and dosing started at 9.30 am), EMG, cortical and hippocampal EEG signals were recorded by telemetry (hereby referred as tEMG, $tEEG_c$ and $tEEG_{hipp}$; Dataquest ART Gold 4.01, DSI). Telemetry data were then analysed with Neuroscore Software v 2.0 (DSI).

Motor activity in the animal homecages (Phenotyper, Noldus Information Technology, Weigeninegen, the Netherlands) was recorded continuously for 24 hours. Video files were recorded from all animals two weeks before PTZ experiment (baseline values) and the day of treatment with vehicle and PTZ 40 mg/kg (an hour before treatment and 23 hours after treatment). Videos were studied by the mean of automated video analysis software (EthoVision XT v 5.1, Noldus). Video synchronization with tEMG, $tEEG_c$ and $tEEG_{hipp}$ was

performed by The Observer XT v 7.0 software (Noldus). For further details of the recording and analysis setup, please refer to (Moscardo E and Rostello C, 2010).

Telemetric recording analysis

Paroxysmal events were categorized as seizures when at least 4 peaks were detectable in an EEG recording (≥ 3 sec; 1 sec minimal interval), characterized by a 1.5 fold amplitude increment than baseline while EEG alterations, shorter than 3 sec, were categorized as spike trains (Figure 38). Since $tEEG_c$ and $tEEG_{hipp}$ presented similar features, we focus our attention and quantitative analysis on $tEEG_c$.

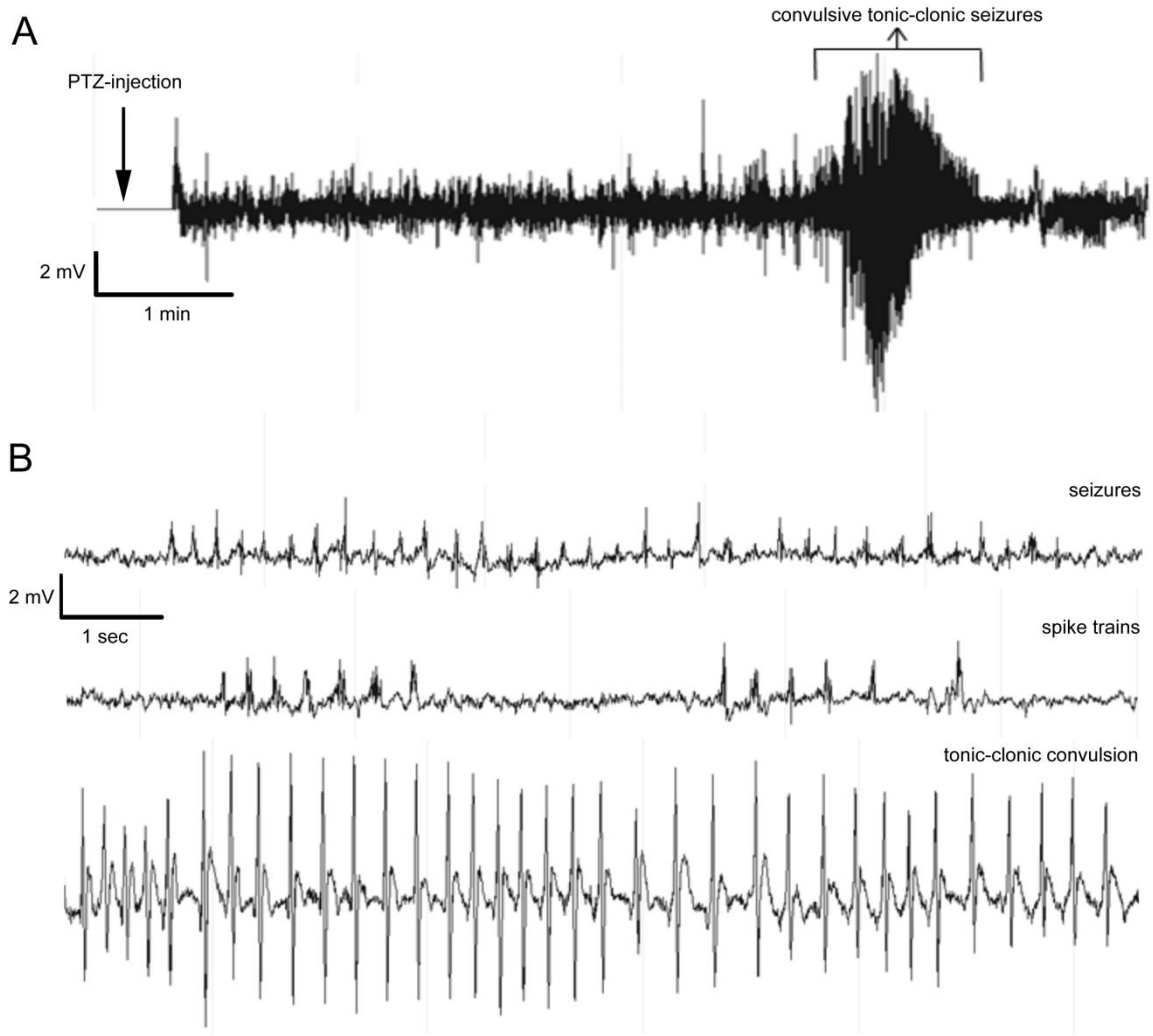


Figure 38: *(**A**) Paroxismal events in cortical t-EEG (t-EEGc) from PTZ treatment to convulsive tonic-clonic event . (**B**) Categorization of paroxismal events in seizure, spike train and convulsive tonic-clonic event.*

Behavioral analysis

For the automatic video-tracking, the following parameters have been analyzed: a) distance moved (the distance traveled of the animal's center-body point); b) movement (the duration of moving of the center-body point); c) velocity (velocity of the animal's center-body point). Analysis was performed over the 23h period and in the first 4h after dosing period.

In order to describe the behavioral PTZ-induced changes, we used a convulsion scoring system (Table I) obtained modifying previous works based on drugs affecting GABA neurotransmission (Beig MI et al., 2007; Lüttjohann A et al., 2009).

Score	Behavioural changes
0	No changes in behavior
1	Sniffing, extensive washing, orientation, tip toe, facial jerking
2	Isolated myoclonic jerks (neck jerks), twitching movements (head nodding)
3	Fully developed bilateral forelimb clonus (in a sitting position)
4-5	Convulsions with clonic and/or tonic-clonic movements, rearing with kangaroo posture (in a sitting position and/or lying on the belly)
6	Convulsions with clonic and/or tonic-clonic movements (lying on the belly and/or wild jumping)

Tab. I*. Scoring system used in order to classify the identified behavioral changes induced by PTZ (modified from Being et al., 2007 and Lüttjohann A et al., 2009).*

Statistical analysis

Statistical analysis of video data was performed using GB-Stat v 7.0 software (Dynamic Microsystems Inc., Silver Spring, MD, USA). One-way repeated measures analysis of variance (ANOVA) was used in the data analysis across days, using the Dunnett post-hoc procedure. For subgroups t-EEG_c differences analysis, Wilcoxon Signed-Rank Test has been used.

Results

Analysis of the Telemetric traces

EEG analysis was based on 6 subjects recorded in three different experimental conditions (baseline, vehicle- and PTZ-injection), in order to have internal controls. No electrical t-EEG_c alterations were detected during baseline and vehicle-injection condition. After PTZ-injection, pathological electrical activity (seizure and spike trains) was detected in all animals, whereas convulsive behavioral events (58.25 ±16.02 sec) were observed in all animals, except rat #2, which has been thus analyzed per se.

Considering animals which showed both seizures and tonic/clonic events (4th to 5th score of the Veliskova's modified scale, see Table I and Figure 39) we identified 2 different sub-groups of animals: an "early convulsion latency" (ECL; 239 ±77.78 sec) and a "late convulsion latency" (LCL; 494 ±50.48 sec) (Tab. II).

a)

Animal	Seizure plus tonic-clonic event duration (s)	Latency (s)
Rat 4	74.00	294.00
Rat 6	66.76	184.00
mean	**70.38**	**239.00**
dvs	5.12	77.78

b)

Animal	Seizure plus tonic-clonic event duration (s)	Latency (s)
Rat 1	33.42	438.00
Rat 3	51.84	536.00
Rat 5	65.26	508.00
mean	**50.17**	**494.00**
dvs	15.99	50.48

Tab. II *Seizure plus tonic-clonic event duration and latency in ECL (a) and LCL (b) sub-groups.*

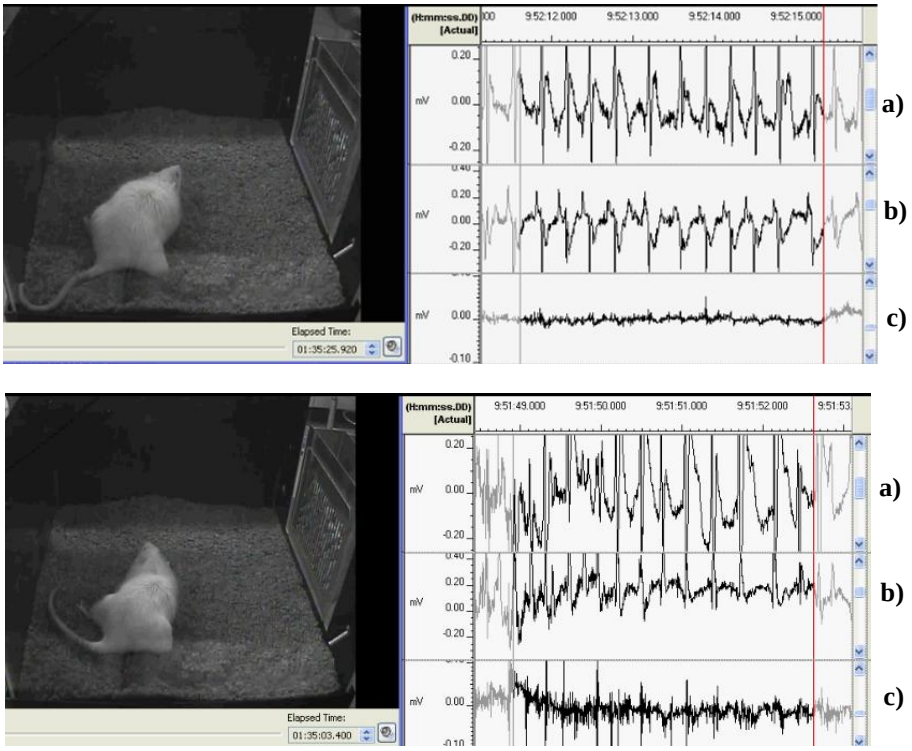


Figure 39: *Example of seizure plus tonic-clonic event in two different moments of convulsion (rat #4; a)EEG$_c$, b)EEG$_{hipp}$, c)EMG).*

No differences have been noted in the electrical alterations frequency and duration before the onset of the behaviorally-detectable tonic-clonic events (Seizures: ECL= frequency: 3 ±1, average duration: 5.74 ±1 sec; LCL= frequency: 5 ±6, average duration: 3.62 ±0.05 sec. Spike trains: ECL= frequency: 10 ±5, average duration: 1.51 ±0.0001 sec; LCL= frequency: 10 ±16.2, average duration: 1.69 ±0.25 sec). On the other hand, electrical alterations frequency after the convulsion-onsets showed significant differences between the two sub-groups (Tab. III; Seizures: ECL= frequency: 122 ±12, average duration: 7.76 ±0.86 sec; LCL= frequency: 23 ±13 ($p \leq .0431$), average duration: 5.65 ±1.95 sec. Spike trains: ECL= frequency: 236 ±190, average duration: 1.56 ±0.13 sec; LCL= frequency: 200 ±143.6, average duration: 1.43 ±0.33 sec).

a)

Animal	Seizure number (post tonic-clonic event)	Mean duration (s)
Rat 4	113	7.15±5.90
Rat 6	130	8.37±6.60
mean	**121.50**	
dvs	12.02	

Animal	Spike train number (post-tonic-clonic event)	Mean duration (s)
Rat 4	102	1.66±0.78
Rat 6	370	1.47±0.51
mean	**236.00**	
dvs	189.50	

b)

Animal	Seizure number (post tonic-clonic event)	Mean duration (s)
Rat 1	8	3.91±0.99
Rat 3	30	5.29±2.57
Rat 5	32	7.75±10.01
mean	**23.33**	
dvs	13.32	

Animal	Spike train number (post-tonic-clonic event)	Mean duration (s)
Rat 1	338	1.20±0.52
Rat 3	45	1.81±0.75
Rat 5	217	1.28±0.62
mean	**200.00**	
dvs	147.24	

Tab. III *Seizure and spike train number detected after the tonic-clonic event in ECL (a) and LCL (b) sub-groups.*

Rat #2, despite the fact that did not show any tonic-clonic event, presented many t-EEG_c alterations (Seizures: frequency: 20, average duration: 4.12 ±1.90 sec; Spike trains: frequency: 261, average duration: 1.57 ±0.50 sec) (Figure 40).

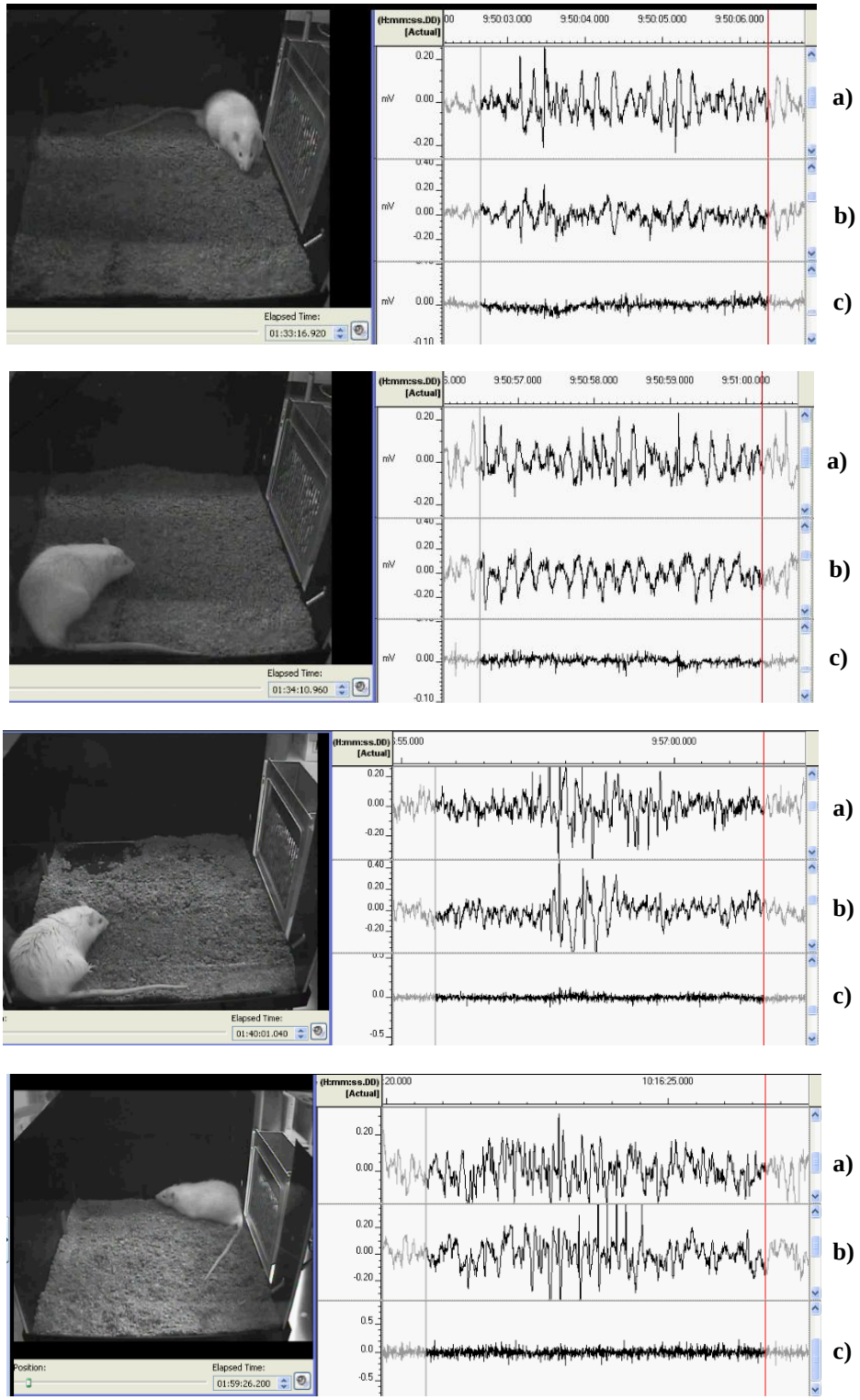


Figure 40: *Examples of t-EEG$_c$ alterations without abnormal behaviour (rat #2; a)EEG$_c$, b)EEG$_{hipp}$, c)EMG).*

Analysis of the videos

In the 23h analysis, no differences were observed in any analyzed parameter between vehicle-injection and baseline; on the other hand, PTZ-treated animals were found to significantly increase the cumulative distance moved (baseline: 70257.27 ±32750.31 m; vehicle-injection: 74609.04 ±27773.22 m; PTZ-injection: 86261.63 ±32297.99 m, p<.01 vs baseline, p<.05 vs vehicle-injection), movement duration (baseline: 7112.28 ±4468.92 min; vehicle-injection: 7229.87 ±4488.62 min; PTZ-injection: 8775.51 ±5005.83 min, p<.05 vs baseline and vehicle-injection) and velocity (baseline: 39.14 ±18.22 m/min; vehicle-injection: 42.55 ±15.73 m/min; PTZ-injection: 47.94 ±17.93, p<.01 vs baseline, p<.05 vs vehicle-injection; see Figure 41).

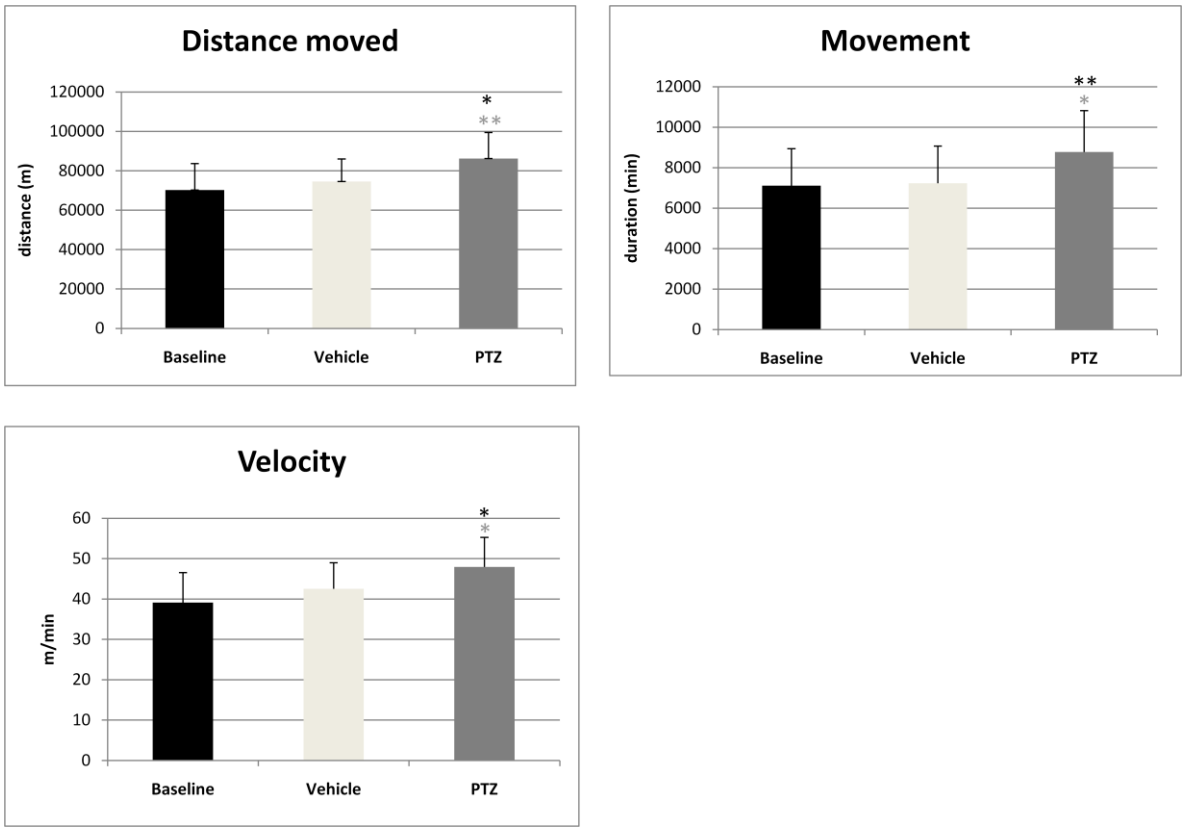


Figure 41: *Motor parameters analysed in 23h video recording (* vs baseline; * vs vehicle;* * p<.05, **p<.01*); Data are expressed as mean±SEM.*

In the 4h analysis, no significant differences were observed between baseline and vehicle-injection groups for movement duration (baseline: 594.11 ±695.11 sec; vehicle-injection: 862.46 ±627.42 sec), whereas a significant increase in distance moved (baseline: 6312.61±4201.03 cm; vehicle-injection: 10111.54 ±3319.22, p<.05) and in velocity (baseline: 3.51 ±2.33 cm/sec; vehicle-injection: 5.62 ±1.91 cm/sec, p<.05). PTZ-injection group showed a significant increase in all considered motor parameters compared with baseline and vehicle-injection groups (PTZ-injection: distance moved 16329.43 ±5521.16 cm, p<.01 vs baseline and vehicle-injection; movement 1691.56 ±760.47 sec, p<.01 vs baseline and vehicle-injection; velocity 9.09 ±3.05 cm/sec, p<.01 vs baseline and vehicle-injection; see Figure 42).

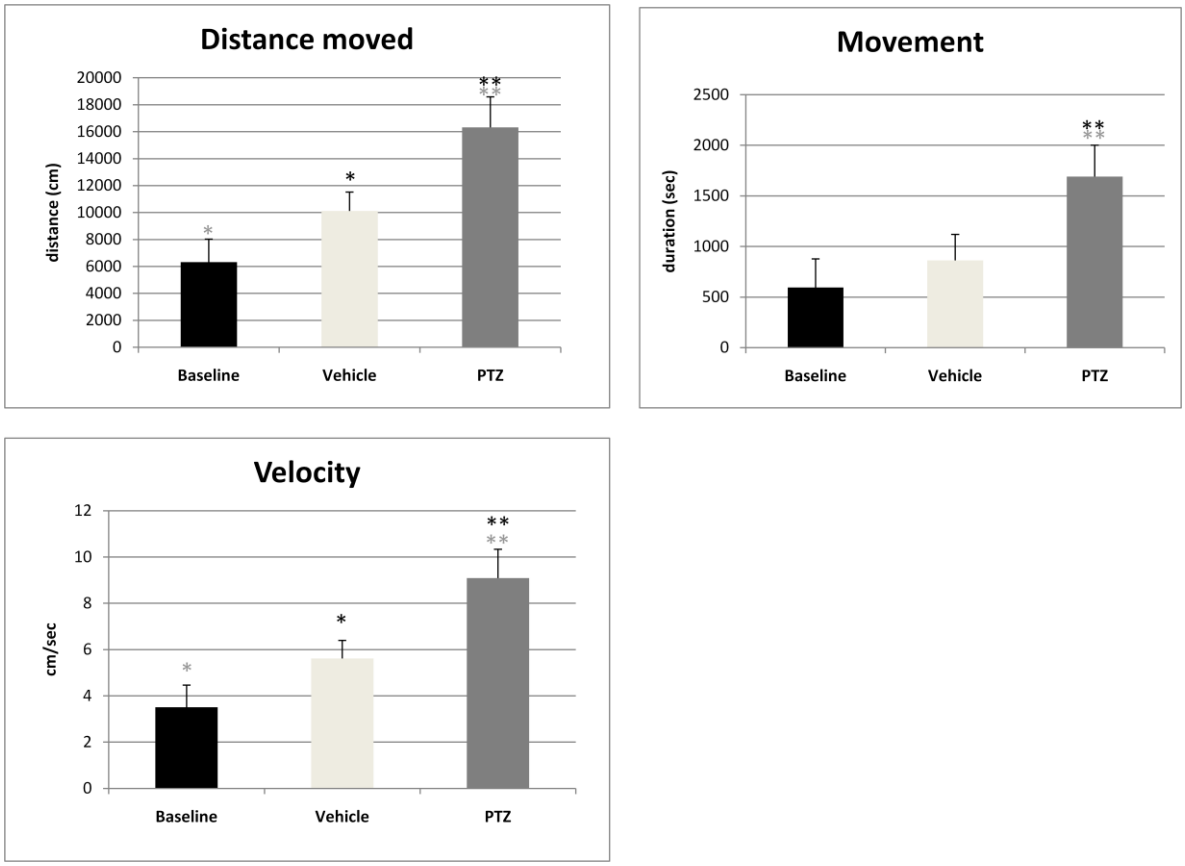


Figure 42: *Motor parameters analysed in 4h (* vs baseline; * vs vehicle); Data are expressed as mean±SEM.*

Besides the behavioral PTZ-induced changes were described using a convulsion scoring obtained modifying previous works based on drugs affecting GABA neurotransmission (Beig MI et al., 2007; Lüttjohann A et al., 2009; Table I).

The behavioural PTZ-induced changes of all 6 rats were analysed. The rat#1 showed abnormal behaviours such as sniffing and facial jerking (score 1) starting by about 2 min after treatment and some isolated myoclonic jerks episodes (score 2) around 5 min after injection; 7.5 min from PTZ administration, the rat showed convulsions with clonic component (score 4) followed by fully developed tonic-clonic movements (score 6; Figure 43).

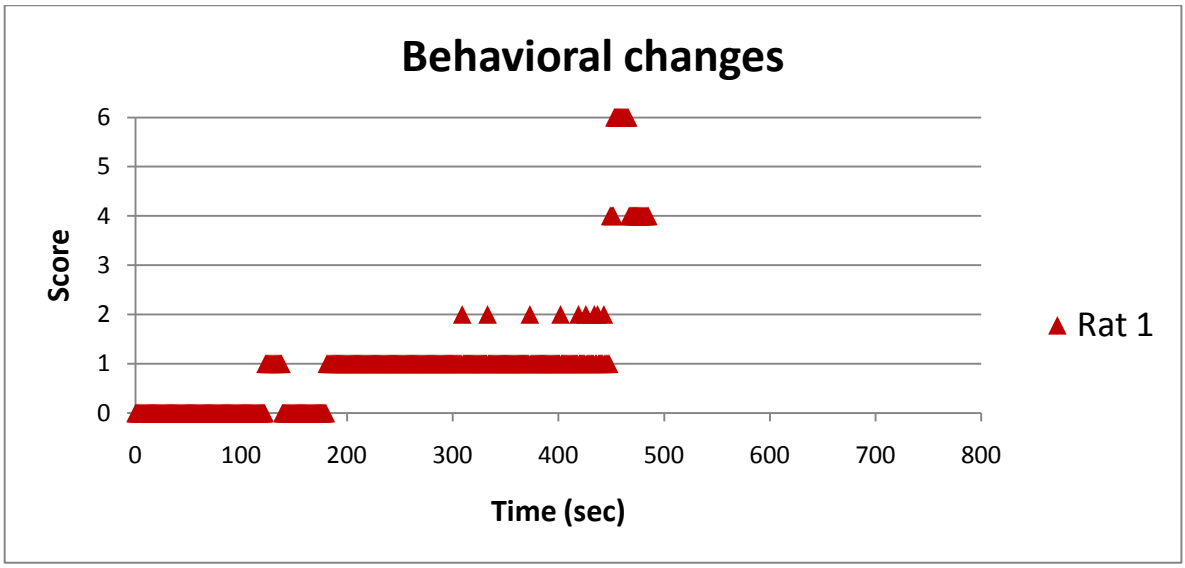


Figure 43: *Behavioural changes observed in rat #1.*

The rat #2 didn't show any tonic-clonic event (scores 4-5,6) but only 2 isolated myoclonic jerks (score 2) at about 0.5 min and 1.7 min after injection and sniffing and orientation behaviours (score 1) at 6 min after treatment for about 2 min (Figure 44).

Figure 44: *Behavioural changes observed in rat #2.*

The rat #3 started with abnormal behaviours (score 1) at about 2.5 min after PTZ injection and these changes were observed for 7 min with several isolated myoclonic jerks (score 2); fully developed tonic-clonic event (score 6) was shown 10 min after injection preceding by a short only clonus of all limbs episode (score 4; Figure 45).

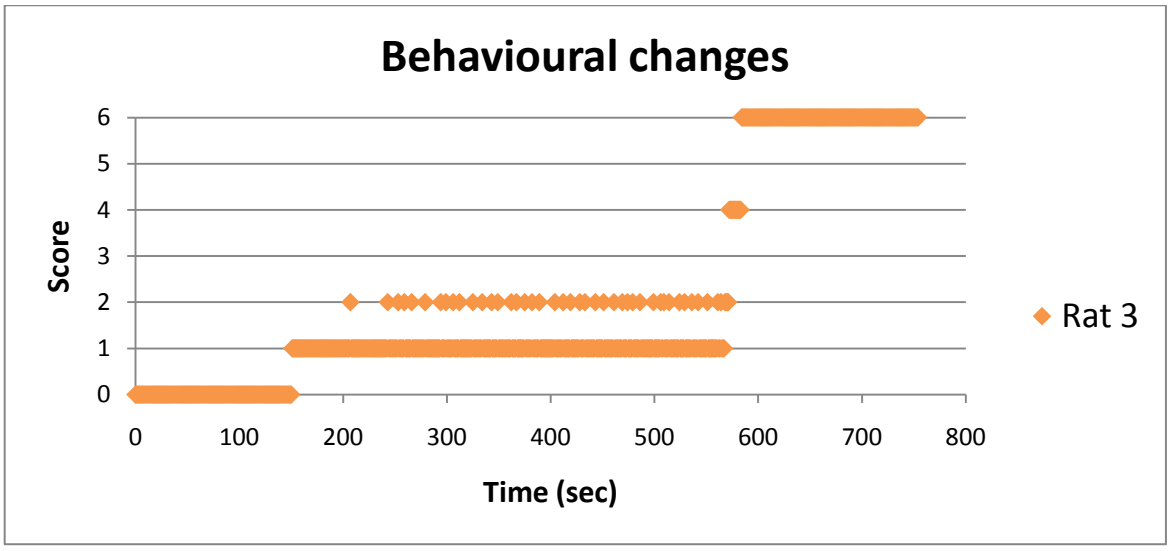


Figure 45: *Behavioural changes observed in rat #3.*

The rat #4 showed one isolated myoclonic jerk (score 2) after only 20 sec and after this one showed abnormal behaviours (score 1) and some isolated myoclonic jerks (score 2) until a tonic clonic movement with suppressed tonic phase (score 4) passing through some

atypical clonic movements and twist of body episode (score 2) about 10 min after PTZ treatment; at the end of convulsions, animal showed fully developed bilateral forelimb clonus (Figure 46).

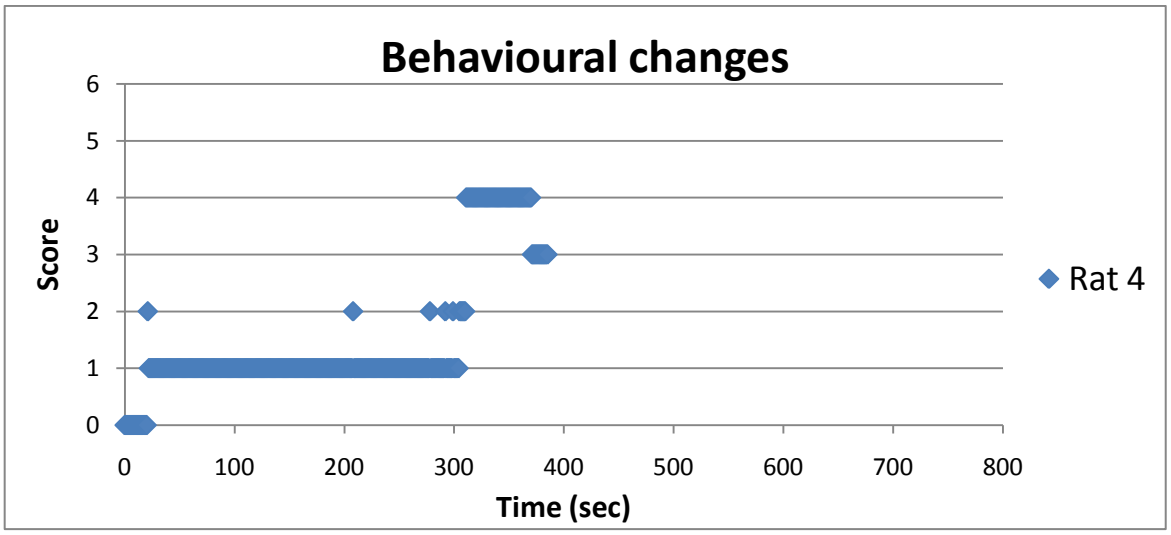


Figure 46: *Behavioural changes observed in rat #4.*

The rat #5 showed abnormal behaviours and several isolated myoclonic jerks (scores 1, 2) starting by about 2 min and until 8.5 min after injection; a fully developed tonic-clonic event (score 6) was shown at about 9 min after treatment and it lasted for about 1 min following by forelimb clonus with a tonic component (score 4,5; Figure 47).

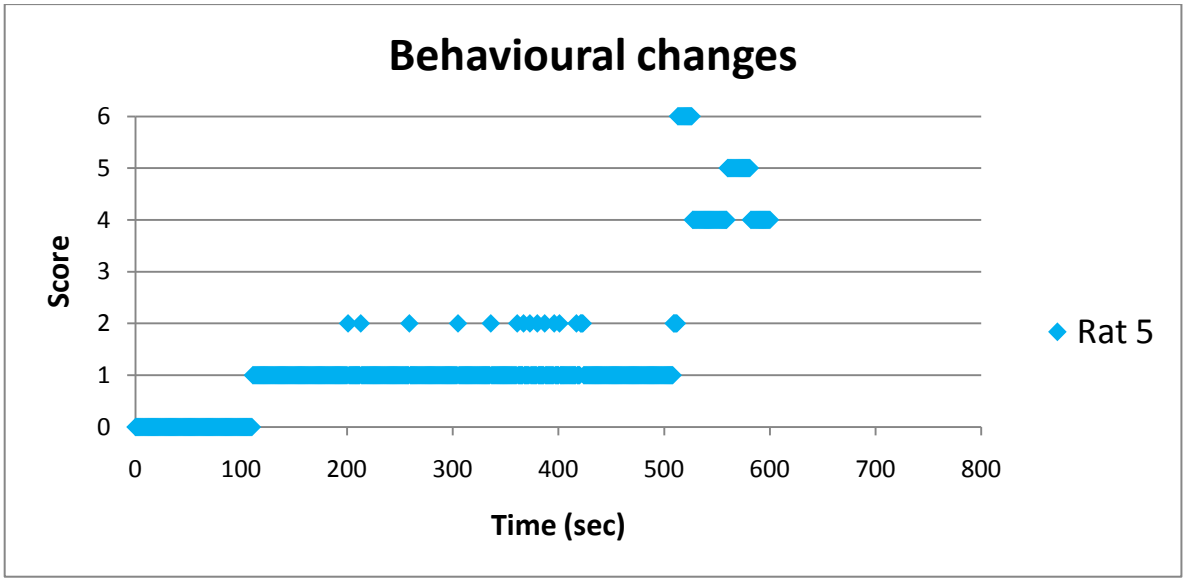


Figure 47: *Behavioural changes observed in rat #5.*

The rat #6 showed abnormal changes and some isolated myoclonic jerks (scores 1, 2) by 1.5 min until 3 min after injection and after these abnormal behavioural events showed a fully developed tonic-clonic event (scores 5, 6; Figure 48).

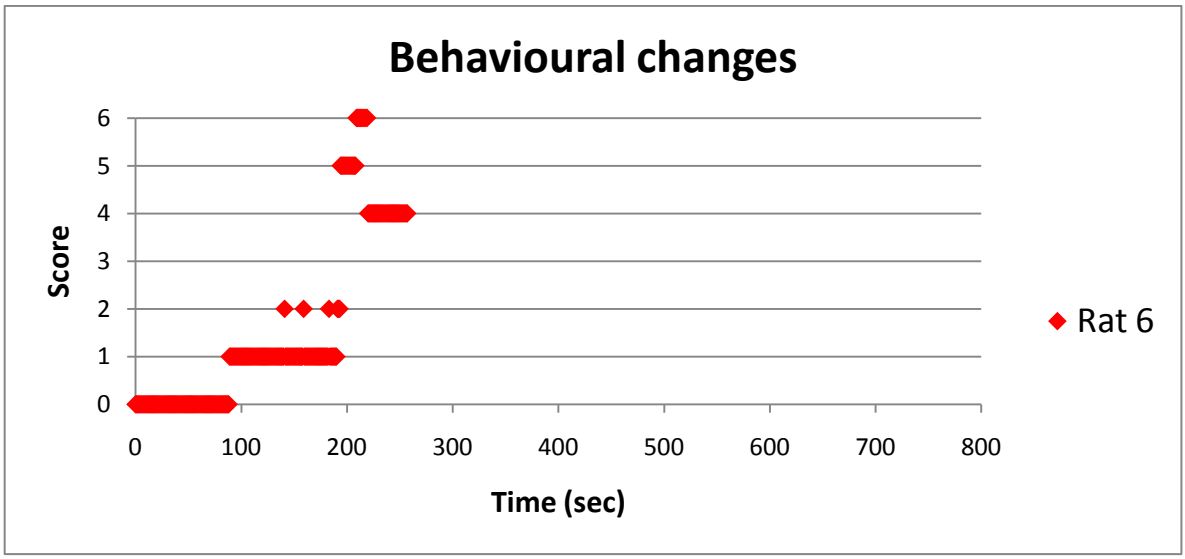


Figure 48: *Behavioural changes observed in rat #6.*

Discussion

We are hereby reporting for the first time that, by the mean of a simultaneous tEEG and behavioural/video analysis, PTZ–injection can be followed by three different pathological conditions: a) electrical alterations (singular spike or spike trains and seizures, with no-behavioural correlates), b) electrical alterations paralleled by tonic-clonic convulsions (behavioural correlates) and, finally, c) post-convulsion EEG alterations (no-behavioural correlates). Given this fact, data based merely on behavioural scoring should be carefully interpreted since the first and the latter condition will be not taken into account. This means an increased risk of false-negative data, that in CNS-safety pharmacology may cause a seizure-liability underestimation.

In absence of behavioural-detectable modifications, PTZ can cause significant EEG alterations

As reported in the results paragraph, Rat #2, despite the fact that did not show any tonic-clonic event, presented many $tEEG_c$ alterations, such as seizures (=20) and spike trains (=261 trains). This case, obviously, will be incorrectly considered negative in a

behavioural-based test, causing an augmented threshold window of the seizure-risk in a safety assessment evaluation.

Before behavioural-detectable modifications, PTZ can cause significant EEG alterations

We should note that, independently to convulsion-latency, four animals up to five showed seizures alterations. The animal showing no seizure before convulsive state was however characterized by a much higher number (> 3.5 fold) of spike trains, confirming the extreme importance of this first window, which is absolutely neglected in a mere behavioural observation.

For an optimal behavioural recognition both the human sensory ability to detect changes in shape by visual observation and the quantitative measures such as speed, distances, precise positions, etc, should be performed. In seizure-convulsion tests, which require long-term observations, observable convulsive events can occur with a low frequency and very rapidly requiring a huge commitment for the observer. For these reasons the video recording is the best way to support and obtain a correct-detailed behavioural scoring analysis, especially in seizure-convulsion tests, because it allows to avoid the inter-observer unreliability applying a cross-review and both the visual identification of behaviours and the automatic analysis of quantitative measures can be obtained (Spruijt BM et al., 1998).

ECL leads to a more severe post-convulsive seizure activity

Considering the two subpopulations based on the latency of convulsions (ECL and LCL), we can observe that animals characterized by shorter latency (ECL) will develop more severe post-convulsive seizure activity, with an augmented seizure frequency. We can thus postulate that the singular susceptibility to PTZ is predictive of the severity of the pathology.

Video-EEG monitoring for seizure-liability assessment

The electroencephalogram (EEG) is the gold standard for seizure detection in the clinical use and is becoming essential also pre-clinically to correctly predict seizure liability (Easter A et al., 2009; Löscher W, 2009). The in-vivo PTZ-induced convulsion is the most commonly used precipitant chemoconvulsant model to assess convulsant risk of NCEs (Easter A et al., 2009). However, it is recognised that the correlation between EEG alterations and behavioural manifestations induced by PTZ progressively increased with

age maturation without reaching a complete correlation (Schickerová R et al., 1984; Pitkanen A et al., 2006; Stanojlović O et al., 2009). On the other hand, EEG used in drug-induced seizure detection allows to capture cases in which seizures occur without behavioural manifestation (false-negative), or conversely, in which convulsion-like behaviours are not seizures (false-positive), increasing the sensitivity and specificity of the pre-clinical animal model (Valentin JP et al., 2009). Therefore, changes in EEG traces can help predict seizure onset and seizure frequency, and may be used as a translatable biomarker for the seizure-liability risk assessment.

Continuous long-term video-EEG monitoring has been used in animal models of epilepsy and pro-convulsion showing to be a critical component of the experimental procedure for differentiating electrical artefacts from EEG seizures, distinguishing EEG seizures from convulsive seizures (Bastlund JF et al., 2004; Williams PA et al., 2006) and to reliably defining the inter-individual variability in seizure frequency, seizure type and occurrence of ciclicity (Goffin K et al., 2007). The reason for combining different tools (e.g., physiological and behavioural) in one experimental model, is to increase the predictive validity of the model generating data that better predict what will occur in humans (Castagnė V et al., 2006; Valentin JP et al., 2009).

Conclusion

This research work demonstrates the importance of applying also in acute pro-convulsion PTZ model an EEG radio-telemetry combined with a synchronised video monitoring model. In particular the video-tEEG model allows to distinguish abnormalities in EEG patterns in the absence of behavioral signs from overt convulsive seizures, also taking into account the inter-individual variability in seizure activity and behavioural manifestation in PTZ model.

Electroencephalography (EEG) provides a direct measurement of brain activity, which is a critical tool in the evaluation of potentially seizure-inducing pharmaceutical candidates and this technology combined with the video monitoring and analysis provide a sensitive and specific pre-clinical tool with a high predictive validity.

b) Video-Telemetry-EEG system and a PK-PD modeling study

Materials and Methods

Six male Sprague Dawley CD^{TM} rats of approximately 7-8 weeks of age were surgical implanted as described above and were selected for this third objective on the basis of their clinical conditions, the quality of telemetric signals and general behaviour. After the surgery required for the installation of the telemetry implants a recovery period of 15 days was respected (Moscardo E and Rostello C, 2010). After this first surgery, all rats were submitted to a second surgical procedure for the chronic cannulation of the femoral vein. The catheter was exteriorized in the interscapular region and rat was equipped with a jacket (Instech Solomon, PA, USA) to allow for protection of the catheter and its connection with the tether (Instech Solomon, PA, USA) (Figure 49).

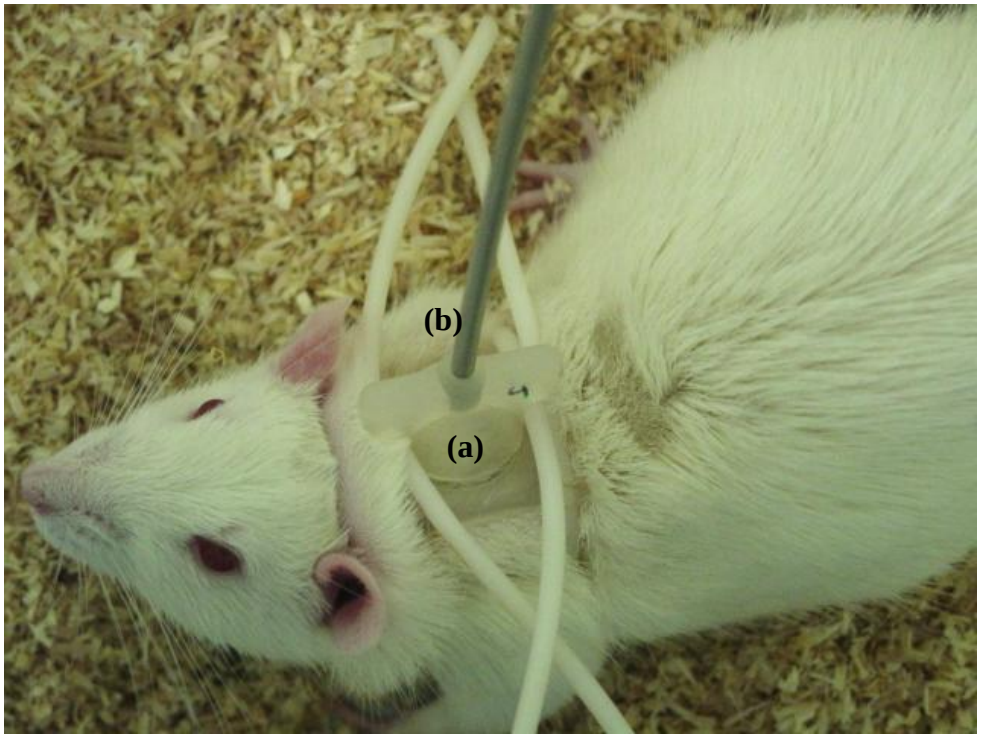


Figure 49: *Animal with jacket (a) connected to metallic theter (b).*

After a post-surgery recovery of 5-6 days all animals were placed in PhenoTyper cages. The catheter was inserted into a 45 cm long metallic tether connected with a balanced harm (Instech Solomon, PA, USA) which was directly fixed to the PhenoTyper cage's wall (Figure 50).

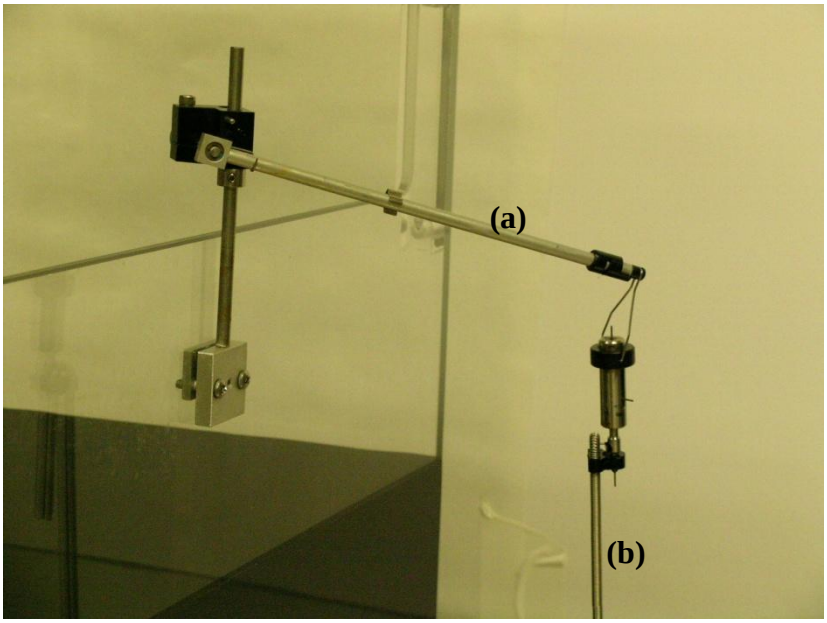


Figure 50: *The balanced harm (a) fixed to the PhenoTyper Cage's wall and the metallic tether (b) for protection of the catheter.*

In order to mantain patency of femoral catheter during whole experimental procedure, it was continuously infused saline with heparin 25UI/mL at 0.2 mL/h by pump (KDS 200, KDS scientific, MA, USA) (Figure 51).

Figure 51: *The PhenoTyper Cages with infusion pump and blood bleeding equipment.*

Study design

In order to obtain time-right plasma concentrations exactly when a seizure-convulsion event was observed, all rats were treated with a test compound known to induce convulsions and videos and telemetric traces were collected continuously starting from one hour pre-treatment until 4 hours post-dose. Blood samples (300 µL blood/time point) from the catheter were collected manually at pre-dose, at three established post-dose time points, and at each occasion when a convulsive episode was observed. The videos obtained were then automatically analysed using the video-tracking software Ethovision XT (Figure 52).

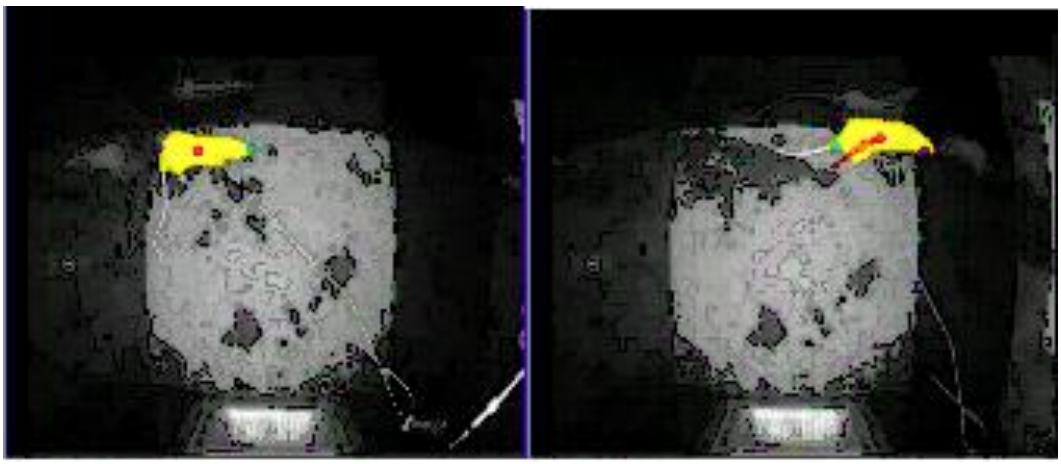


Figure 52: *The Video tracking analysis with Ethovision XT.*

Results and Discussion

All blood samples required were collected successfully including on the occasion of convulsion events. The videos analyzed by the Ethovision XT software was able to track animals also in presence of the additional equipment required for the bleeding (i.e., jacket, tether and balance harm) with no interferences with the telemetric recordings, confirming that this set up did not compromise in any way the behavioral tracking made by the software (Figure 52).

The Video-tEEG system can be successfully combined with a simple tether-based system for sequential blood bleeding in the laboratory rat, without compromising the functionality of the whole equipment set-up during the recording phase and the efficiency of the automatic video-tracking analysis. The combined video-tEEG and blood bleeding systems can be used in pre-clinical investigations to better define the PK/PD profile of drug-induced convulsions or seizures, allowing for safer investigations in the clinic. The developed combined system could potentially find further application in many different research areas, allowing integration of good quality behavioral, physiological and pharmacokinetic data and enhancing data interpretation.

Final Remarks and Conclusions

All the experiments described into this research work 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.

The results of this research confirm that an integrated Video-Telemetric Electroencephalography (video-tEEG) system which allows the simultaneous and synchronised recording of video, cortical, hippocampus electroencephalograms and electromiogram in freely moving rats, can find innovative applications in different research areas, where is of particular interest to obtain simultaneously physiological and behavioural data.

In particular, the Video tEEG system could be applied in routine research areas. For example it could be used for analyzing the influence of housing environmental conditions, post-operative care procedures and training procedures on the experimental collected data and results. In the first part (Part I) of this research the system was used in order to evaluate the relations between different environmental conditions, behavioural parameters and “brain activity” (sleep pattern and Theta activity). In particular the set up was used to answer to this question: “Can experimental procedures as housing condition and/or cleaning procedure affect or alter the reliability of data?”. The video-tEEG model allows to demonstrate how it is important to check any possible influence of test conditions and also the routine laboratory procedures like cleaning, in order to ensure the reliability and daily repeatability of experimental data.

A second interesting application is related to the analysis of the relationship between electrical seizures and behavioural changes/convulsions, where the model could contribute in refining the conventional models (i.e., tests of chemical and electrical induction of seizures) in order 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 evaluated during the second part of this research (Part II), where the Video tEEG system was used to investigate physiological and behavioural correlations following administration of a pro-convulsive reference compound (Pentylentetrazole) in rats. In this area the system should allow the identification of the seizure-inducing potentiality, until convulsive threshold doses and the establishment of relationship between

behavioural and clinical signs preceding and accompanying convulsive episodes. This research work demonstrates the importance of applying also in acute pro-convulsion PTZ model an EEG radio-telemetry combined with a synchronised video monitoring model. In particular the video-tEEG model allows to distinguish abnormalities in EEG patterns in the absence of behavioural signs from overt convulsive seizures, also taking into account the inter-individual variability in seizure activity and behavioural manifestation in PTZ model. Electroencephalography (EEG) provides a direct measurement of brain activity, which is a critical tool in the evaluation of potentially seizure-inducing pharmaceutical candidates and this technology combined with the video monitoring and analysis provide a sensitive and specific pre-clinical tool with a high predictive validity.

In this research, it was also evaluated the feasibility for the integration of a Video-telemetry Electroencephalography (video-tEEG) system with a set up for sequential blood bleeding in the laboratory rat (Part III). The successful developed combined system shows the possibility to obtain an integration of behavioral, physiological and pharmacokinetic data, enhancing data interpretation. This combined model is considered potentially extremely helpful for a reliable pharmacokinetic/pharmacodynamic (PK/PD) modeling of behavioral and EEG changes following the administration of a new candidate drug by a time-right identification of plasma concentrations.

Telemetric waveforms and the activities of animals collected with this integrated system provide unprecedented insight into the correlations between phonotypical-behavioral 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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