Full Study Report
Second Version
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EVALUATION OF ACUTE EFFECTS OF AC101 COMPARED TO BASMISANIL, MRK016 AND DIAZEPAM ON qEEG USING TELEMETRY EEG RECORDING IN NAÏVE CONSCIOUS RAT
– NON-GLP STUDY –

Biotrial Pharmacology Study Code:
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Sponsor Study Reference Number:
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Study Dates	
Study initiation date	20 DEC 2022 *(date of Study Director's signature of the Study Plan*)
In-life experimental starting date	05 DEC 2022
In-life experimental completion date	20 JAN 2023
Study completion date	Equals the date of Study Director's signature of the Final Study Report
Objectives	To evaluate the acute effects of AC101 compared to Basmisanil, MRK016 and Diazepam on EEG power spectrum in the chronically implanted conscious rat
Distribution	Damona Pharmaceuticals Biotrial Archives

CONFIDENTIALITY CLAUSE

STUDY DIRECTOR'S STATEMENT

The study was not GLP.

However, concerning experiments performed at Biotrial, analysis and reporting, studies are carried out in a quality-driven GLP-certified laboratory to ensure optimum environmental control and traceability.

All data and results generated during the study are the property of the Sponsor.

_____

Kevin Carvalho Date (*DD/MMM/YYYY*): _ _ / _ _ _ / _ _ _ _
Study Director

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LIST OF ABBREVIATIONS

Abbreviation	:	Definition
AAALAC	:	Association for the Assessment and Accreditation of Laboratory Animal Care
a.m.	:	Morning (*ante meridiem*)
ANOVA	:	Analysis of variance
bid	:	*bis in die*
°C	:	Degree Celsius
cm	:	Centimetre
CR2EA	:	Comité de Réflexion Ethique en Expérimentation Animale (Biotrial Ethics Committee)
EEG	:	Electroencephalography
e.g.	:	Exempli gratia (for example)
EMG	:	Electromyogram
EU	:	European Union
FFT	:	Fast Fourier Transform
G	:	Gram
GLP	:	Good Laboratory Practice
h	:	Hour
Hz	:	Hertz
i.e.	:	That is (*id est*)
ip	:	Intraperitoneal
kg	:	Kilogram
µg	:	Microgram
mg	:	Milligram
mL	:	Millilitre
mm	:	Millimetre
NA	:	Not applicable
No	:	Number
OECD	:	Organisation for Economic Co-operation and Development
p.m.	:	Afternoon (*post meridiem*)
qEEG	:	Quantified Electroencephalography
sc	:	Subcutaneous
SD	:	Standard Deviation
SEM	:	Standard Error of the Mean
SOP	:	Standard operating procedure
T	:	Timebin
USA	:	United States of America

1 SUMMARY

1. The objective of this study was to evaluate the acute effects of AC101 (5 or 15 mg/kg, ip) compared to Basmisanil (5 mg/kp, ip), MRK016 (3 mg/kg, ip) and Diazepam (2 mg/kg, ip) on electroencephalography (EEG) power spectrum in the chronically implanted conscious rat, using telemetry.

2. The telemetered male Sprague-Dawley rats used in this study were naïve animals and were equipped for telemetry EEG, electromyography (EMG), and actimetry recordings. The experiments took place during the dark phase (12h/12h light cycle, dark 8:00 *a.m.* to 8:00 *p.m.*). Results for 7 animals are presented because an outlier animal for EEG signal was excluded for data analysis (see Section 4.5, Deviation No. 3).

3. For the first telemetry session, after 2h of continuous recording before treatment, all telemetered animals were administered with an intraperitoneal administration of saline. For the following telemetry session, all telemetered animals were administered with an intraperitoneal administration of vehicle [Methyl cellulose 0,5% in water for injection], or Basmisanil (5 mg/kg, ip), MRK016 (3 mg/kg, ip), Diazepam (2 mg/kg, ip), or AC101 (5 or 15 mg/kg) according to a crossover design. A minimum washout period of 48h was respected between each telemetry sessions per animal.

4. EEG, EMG and actimetry signals were continuously recorded and analysed from 2h before each administration to 4h after each administration for: 1) frequency components of the quantitative EEG (qEEG) (total power, absolute and relative power in the delta band [1, 4 Hz[, theta band [4; 8 Hz[, alpha band [8, 14 Hz[, beta band [14, 32 Hz[, and gamma band [32, 49 Hz] U [51, 80 Hz]; 2) a spectral EEG analysis and 3) locomotor activity from actimetry recordings.

5. Administration of Basmisanil, MRK016, Diazepam and AC101 were associated with the following notable effects when compared to vehicle:
   - An overall dose-dependent increase in total power of the EEG after administration of AC101 at 15 mg/kg and Diazepam.
   - A 4h post-dose increases of the power for frequencies between 11 to 49 Hz (corresponding to Alpha 2 and Gamma 1 bands, respectively) after administration of AC101 at 15 mg/kg and Diazepam.
   - No meaningful effects of MRK016 and Basmisanil on EEG spectrum.
   - Effect of saline or vehicle administration was similar on EEG power over time.
   - The locomotor activity confirmed the inverted cycle and low activity 1h after the light were turned off.

6. In conclusion, administration of AC101 dose-dependently increased the overall power of the EEG over time in the rat, measured with telemetry and using a frequency component analysis and a spectral analysis. The highest dose 15 mg/kg significantly increased the power in higher frequencies (Alpha 2 to Gamma 2 bands [11 – 80 Hz]) until the end of the 4h of recording. The effects are the same as those observed for Diazepam but with a slower and longer dynamic.

2 INTRODUCTION

Electroencephalography (EEG) measures integrated brain activity. Electroencephalograms are composed of electrical signals of different frequencies [1]. EEG has gained prominence as an ideal biomarker as it is continuous, objective, repeatable, reproducible, sensitive and, depending of the paradigm, translatable from animals to humans. EEG provides both safety and pharmacodynamics measurements, enabling a deeper understanding of the pharmacological effects of the drug early in its development. Using radiotelemetry techniques, EEG can be monitored in freely moving animals, thereby minimizing any artefacts due to stress.

DAMONA has developed a new molecule, AC101, a positive allosteric modulator of the α5-GABAA receptors and is willing to determine the EEG signature related to administration of the molecule in rats.

3 OBJECTIVES

The purpose of this study was to evaluate the acute effects of AC101 compared to Basmisanil, MRK016 and Diazepam on EEG power spectrum in the chronically implanted conscious rat.

In this study, EEG and EMG were analysed in the conscious unrestrained rat, after surgical implantation of telemetric devices. Using this approach, EEG and EMG were continuously monitored after single ip administrations of AC101 (5 or 15 mg/kg), Basmisanil, MRK016 and Diazepam in freely moving animals.

4 MATERIALS AND METHODS

The study was conducted in AAALAC accredited facilities under EU and French animal welfare regulations for animal use in experimentation (European Directive 2010/63/EU and French decrees and orders 2013-118 of February 1st, 2013, and 2020-274 of March 17th, 2020). This experimental project is approved by the Biotrial Ethics Committee "Comité de Réflexion Ethique en Expérimentation Animale (CR2EA) (registered by the "Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation" (French ministry of higher education, research and innovation) under No. 67).

4.1 Animal management

4.1.1 Description

The study was carried out using 12 male Sprague-Dawley rats (in order to include 8) (Janvier Labs, Saint Berthevin, France), 6-7 weeks old, weighing 256-304 g on the day of surgical implantation (see Section 4.5, Deviation No. 1).

4.1.2 Housing conditions

The animals were housed in groups of 2-4 in polysulfone cages (floor area = 1500 cm²) under standard conditions: room temperature (22±2°C), light/dark cycle (12h/12h, dark 8:00 *a.m.* to 8:00 *p.m.*), air replacement (15-20 volumes/h), water and food (Safe® A04, Safe, Augy, France) *ad libitum*.

The chronically instrumented rats were individually housed after surgery until the end of the study and were identified by an ear tattoo.

The animals were allowed to habituate to environmental conditions for at least 5 days prior to experimentation.

4.1.3 Choice of species

The rat has been chosen to evaluate the effects of the Test Items on the EEG as it is the most practical and commonly used species for EEG evaluation of drug effect on the central nervous system.

4.1.4 Surgical procedure

Surgery was performed on 12 rats in order to include at least 8 implanted rats in the study.

Animals were not fasted before surgery.

Surgery was performed under isoflurane anaesthesia (5% isoflurane/air for anaesthesia induction and 1.5-3% isoflurane/air for anaesthesia maintenance).

The two EEG leads of the telemetry implant (HD-S02 implant, Data Sciences International, St Paul, MN, USA) were fixed on the skull with screws and dental acrylic at the following stereotaxic coordinates: Antero/Posterior: +2.0 mm to Bregma, medial/lateral: +1.5 mm to midline for negative lead; and Antero/Posterior: -7.0 mm to Bregma, medial/lateral: -1.5 mm to midline for positive lead.

In addition, EMG leads were fixed in contact with the dorsal muscles of the neck. EEG and EMG leads were subcutaneously tunnelled.

The telemetry transmitter itself was placed subcutaneously along the animal flank and attached with non-absorbable sutures. The skin incisions were then closed with a re-absorbable suture.

Post-surgical analgesia was ensured by subcutaneous administration of buprenorphine (10-50 µg/kg, sc) bid for 2 days (including surgery day) and meloxicam (1-2mg/kg, sc) od for 3 days (including surgery day). At least 10 days were allowed to ensure complete recovery from the surgery before first administration. During this recovery period, the animals were weighed and observed daily, and the sutures were examined and disinfected if needed with antiseptic solution (povidone-iodine) if needed.

Quality control of physiological signals (visual inspection of the signal and spectral analyse of EEG and EMG on short recording) were performed few days prior the first dosing to select animal to be included in the study.

4.1.5 Fate of animals

At the end of the experiments, the surviving animals were not sacrificed and were returned to the stock, pending another experimental cycle.

Two moribund animals were observed during the study and had to be sacrificed (see Section 4.5, Deviation No. 2).

4.2 Drugs

4.2.1 Vehicle

Vehicle	
Name/code	Methyl cellulose 0,5%
Reference/manufacturer and supplier	Sigma-Aldrich M0262
Method of preparation for vehicle formulation	Methylcellulose was dissolved in purified water The preparation procedure is documented in the study data.
Stability in storage conditions	5±3°C for 8 days

4.2.2 Test Item

AC101 (batch No. PM-II-51) was provided by the Sponsor and stored at Biotrial Pharmacology according to the conditions specified by the Sponsor. The Sponsor certified that the AC101, which was to be tested in the present study, was the Test Item that was sent to Biotrial Pharmacology.

Test Item		
Name/code	AC101	
Manufacturer / Supplier	University of Wisconsin - Milwaukee	
Batch Number	PM-II-51	
Test Item characterization		
Correction factor	No correction factor was applied.	
Storage conditions	☒ Room Temperature	☐ 5±3°C
	☐ Protected from light	☐ Other, specify:
Stability in storage conditions	More than 2 years	
Sample Aliquot	A sample of the batch of the Test Item used for the study was taken and kept separately until finalisation of the Study Report/Study.	
Quantity required	A minimum of 160mg of Test Item was required to carry out the study.	

Test Item formulation in vehicle		
Type of formulation	☐Suspension	☒ Solution
Concentration	Concentration 1: 5 mg/kg Concentration 2: 15 mg/kg	
Storage conditions of formulations	☐ Room Temperature	☒ 5±3°C
	☐ Protected from light	☐ Other, specify:
Stability of formulations	Formulations in the concentration range of 0.5 mg/mL to 100 mg/mL were confirmed as stable for up to 8 days when stored at storage conditions	
Method of preparation for Test Item formulations	The Test Item preparation was done by the Test Facility according to the preparation instructions provided by the Sponsor prior to the start of the study.	

4.2.3 Test Item surplus

Before the end of the Study (*i.e.*, the signature of the Study Report by the Study Director), any surplus Test Item was returned to the Sponsor.

4.2.4 Reference Item 1

Reference Item	
Name/code/CAS	Basmisanil (CAS 1159600-41-5)
Reference and supplier	MedChemExpress HY-16716
Correction factor	No correction factor was applied.
Formulation procedure	The Reference Item was prepared in Methyl cellulose 0,5% using mortar and pestel until homogeneous suspension is obtained. Finally, it was constantly stirred to ensure drug did not drop out of solution. The preparation procedure is documented in the study data.
Stability and storage conditions	Stability in solution unknown. Was freshly prepared before administrations.

4.2.5 Reference Item 2

Reference Item	
Name/code/CAS	MRK016 (CAS 342652-67-9)
Reference and supplier	MedChemExpress HY-100370
Correction factor	No correction factor was applied.
Formulation procedure	The Reference Item was prepared in Methyl cellulose 0,5% using mortar and pestel until homogeneous suspension is obtained. Finally, it was constantly stirred to ensure drug did not drop out of solution. The preparation procedure will be documented in the study data.
Stability and storage conditions	Stability in solution unknown. Was freshly prepared before administrations.

4.2.6 Reference Item 3

Reference Item	
Name/code/CAS	Diazepam (CAS 439-14-5)
Reference and supplier	Sigma-Aldrich D0899
Correction factor	The dose of Reference Item is expressed as salt. No correction factor was applied.
Formulation procedure	The Reference Item was prepared in Methyl cellulose 0,5% using mortar and pestel until homogeneous suspension is obtained. Finally, it was constantly stirred to ensure drug did not drop out of solution. The preparation procedure was documented in the study data.
Stability and storage conditions	Stability in solution unknown. Was freshly prepared before administrations.

4.3 Study design

4.3.1 Choice of the route of administration

The intraperitoneal route of administration was used to evaluate the effects of the Test Item on the EEG power spectrum as it was the only usable route due to AC101 fast metabolism in rats.

4.3.2 Administration protocol

After 2h of continuous recording before treatment, saline, the vehicle, the Test Item or one of the Reference Items were administered by ip using a cross-over design (at the exception of the first session) with n=8 rats and 7 telemetry sessions per animal. A minimal washout period of 48h was respected between two sessions.

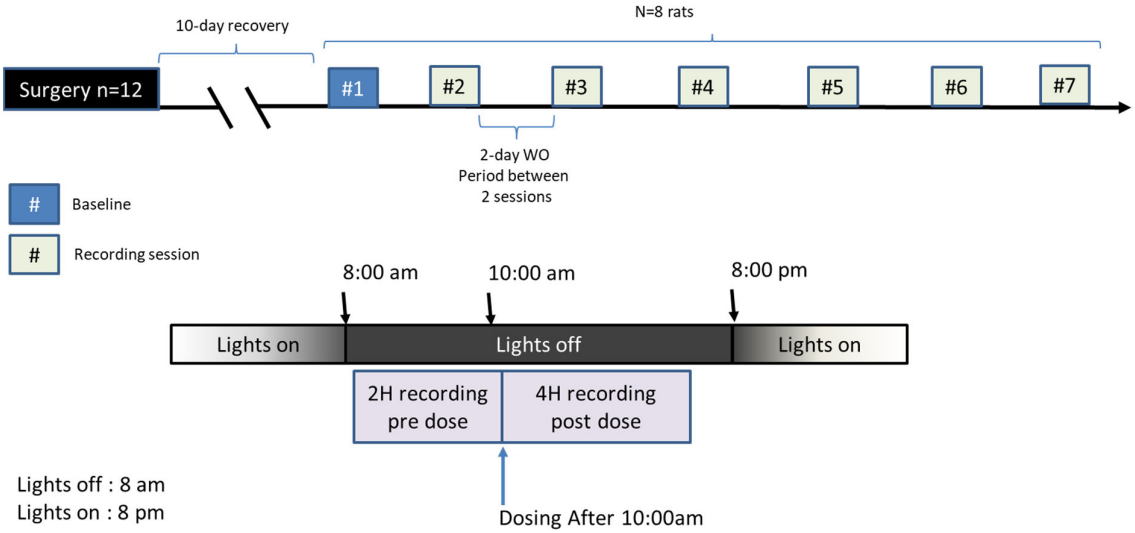
Each telemetered rat received on separate occasions:

1. Vehicle (5 mL/kg, ip)
2. AC101 (5 mg/kg, ip)
3. AC101 (15 mg/kg, ip)
4. Basmisanil (5 mg/kg, ip)
5. MRK016 (3 mg/kg, ip)
6. Diazepam (2 mg/kg, ip)

Animals	Session 1 (Saline)	Session 2	Session 3	Session 4	Session 5	Session 6	Session 7
Rat No. 9	Saline	AC101 (5 mg/kg)	Basmisanil (5 mg/kg)	Diazepam (2 mg/kg)	AC101 (15 mg/kg)	MRK016 (3 mg/kg)	Vehicle
Rat No. 2 / Rat No. 1*	Saline	Basmisanil (5 mg/kg)	AC101 (15 mg/kg)	AC101 (5 mg/kg)	Vehicle	Diazepam (2 mg/kg)	MRK016 (3 mg/kg)
Rat No. 3	Saline	AC101 (15 mg/kg)	Vehicle	Basmisanil (5 mg/kg)	MRK016 (3 mg/kg)	AC101 (5 mg/kg)	Diazepam (2 mg/kg)
Rat No. 4	Saline	Vehicle	MRK016 (3 mg/kg)	AC101 (15 mg/kg)	Diazepam (2 mg/kg)	Basmisanil (5 mg/kg)	AC101 (5 mg/kg)
Rat No. 6	Saline	MRK016 (3 mg/kg)	Diazepam (2 mg/kg)	Vehicle	AC101 (5 mg/kg)	AC101 (15 mg/kg)	Basmisanil (5 mg/kg)
Rat No. 7	Saline	Diazepam (2 mg/kg)	AC101 (5 mg/kg)	MRK016 (3 mg/kg)	Basmisanil (5 mg/kg)	Vehicle	AC101 (15 mg/kg)
Rat No. 10	Saline	AC101 (5 mg/kg)	AC101 (15 mg/kg)	Diazepam (2 mg/kg)	MRK016 (3 mg/kg)	Vehicle	Basmisanil (5 mg/kg)
Rat No. 12	Saline	Basmisanil (5 mg/kg)	Vehicle	MRK016 (3 mg/kg)	Diazepam (2 mg/kg)	AC101 (15 mg/kg)	AC101 (5 mg/kg)

*Due to postoperative complications, rat No. 2 was replaced by rat No. 1 starting from Session 3 (see Section 4.5, Deviation No. 2). The cross-over design was respected.

The design of the study is summarized in Figure below:



The volume of administration was 5 mL/kg body weight for intraperitoneal administration.

The doses of Test Item have been chosen by the Sponsor according to the known pharmacological profile of this compound.

The Study Director was required to inform the Study Monitor of all adverse events which could impact on dosing and study results.

4.3.3 Experimental procedure

The telemetry and data acquisition system from Data Sciences International (St Paul, MN, USA) was used. During the experimental session, the animals were individually housed in cages placed on the receiver panel. The EEG and EMG signal were monitored, analysed, and stored on the hard drive of a compatible personal computer using HEM software (version 4.4, Notocord, France).

EEG and EMG were continuously recorded from 2h before each administration to 4h after each administration. The analysis was performed over the 6h.

The EEG recording session was performed during the active phase (*i.e.*, dark phase: between 8 *a.m.* and 8 *p.m.*) in order to minimize sleep EEG which could lead to misinterpretation of the drug effects.

Automatic sleep scoring algorithm split the recording into sleep and wake episodes. This algorithm relies on spectral components of EEG and EMG. In order to reduce variability within the dataset, spectral analysis of the EEG was conducted on wake periods only.

4.3.4 Measured and calculated parameters

4.3.4.1 Parameter estimation

Data analysis was performed by the Biotrial Core Lab.

Quantified electroencephalography (qEEG) was measured and provided information about the frequency components of the EEG signal.

Spectral analysis of EEG was performed on non-overlapping 4-second signal segments (= epochs) using Fast Fourier Transform (FFT). For each epoch we calculate a power spectrum on the range [0-80 Hz] with a 0.25-Hz resolution. Power spectra were obtained by using the product of the complex spectra by the complex conjugate spectra. The same is done on EMG signal. On the same 4-second windows we quantify the movement (count for actimeter embedded in the acquisition implant). All these pre-processing parameters are used to create a 3-state hypnogram (wake, sleep or artifact) then extract final parameter.

Automatic sleep staging is performed by a Matlab script that run a decision tree to classify each epoch. It relies on EEG amplitude, EEG power in low frequency band, actimetry and EMG in [10-45 Hz] band. Decision thresholds used by decision tree are adjusted manually for each animal. The same thresholds are used for all recordings of a giving animal. Each 4-second epoch receives a label “wake”, “sleep” or “artifact”.

For each epoch, the power on each standard qEEG frequency band and sub-band were extracted (sum of power spectrum values within the band). We also estimated the total power on the full EEG band and used it to compute the relative powers: power on a given frequency (called absolute power) divided by the total power. Working with relative powers reduces the variability between animals. Thanks to wireless acquisition system and design of our facilities there’s no clear noise at 50Hz however to avoid any issue due to this type of noise we reject the band]49-50 Hz [from analysis. The bands of interest are described below.

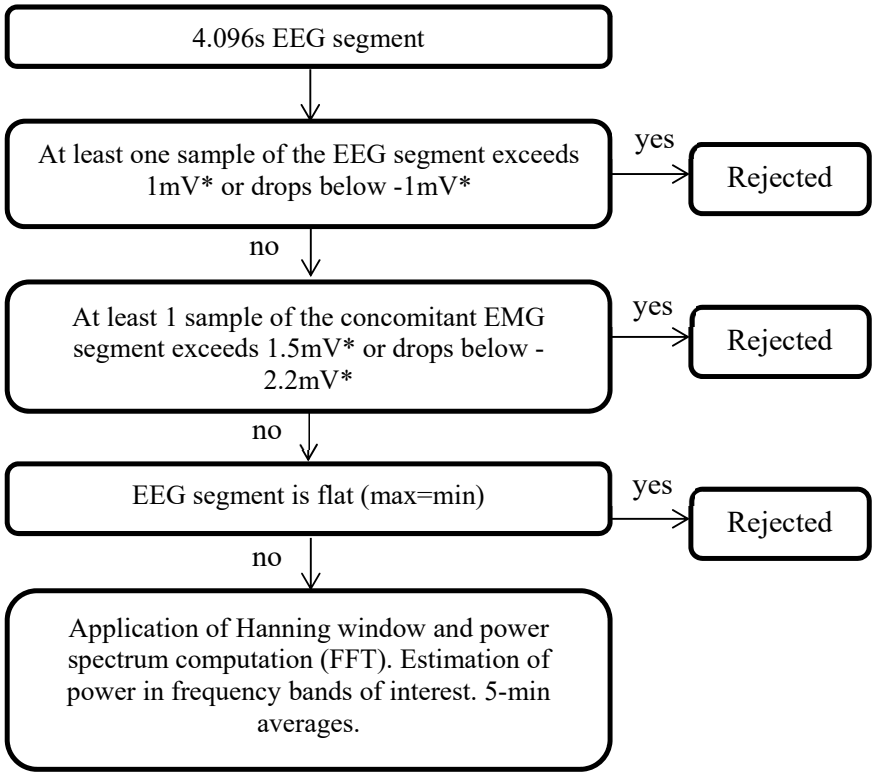
For each recording and band, we average the power is calculated using “wake” epoch only on 5 time points: one 2h-timebins at baseline and four 1h-timbins at post-dose. In addition to the work band by band, the grand average spectra using 0.5 Hz bins from 1 to 80 Hz from “wake” epoch was calculated and graphically smoothed. It leads to figure that help to visualize drug effects in a similar way than in published study describing effect of Basmisanil on human being [2].

Activity is quantified by actimeter and the average number of counts per minute is provided for each timepoint.

Band Name	Definition (Hz)
Delta	[1, 4[
Theta	[4, 8[
Theta 1	[4, 6.5[
Theta 2	[6.5, 8[
Alpha	[8, 14[
Alpha1	[8, 11[
Alpha2	[11, 14[
Beta	[14, 32[
Beta1	[14, 18[
Beta2	[18, 32[
Gamma	[32, 49] U [51, 80]
Gamma1	[32, 49]
Gamma2	[51, 80]
Total	[1, 49] U [51, 80]

4.3.4.2 Epochs selection

An EEG epoch was rejected from any further analysis according to the following decision tree:



Therefore, five-minute segments could last less than 299 seconds due to epoch rejections. When all 4.096-second epochs for a segment were rejected, the average value on the 5-minute segment was not estimated. EMG signal data was used in the rejection process only.

The 5-minute segment included in a time points was averaged to provide a single value for each time point and each EEG band.

4.4 Data processing and statistical analysis

Test parameters were analysed at Biotrial using SAS software (version 9.4).

For each frequency band, individual data listings, descriptive statistics (n, mean, SD, SEM, minimum, maximum) for raw data for each time bin, changes from baseline and percent changes from baseline for each time-matched bin as well as graphs illustrating means (± SEM) over time (2h timebins for baseline and 1h timebins post-dose) by treatment group for changes from baseline for each time-matched bin were provided.

EEG power spectrum will be displayed graphically illustrating means for percent changes from baseline and raw data ratio values for each time-matched bin.

The significance threshold was 5%, except for the (treatment x Time) interaction (alpha = 10%).

For comparison of Vehicle versus other items (except Saline) and for absolute and relative power and for each frequency band, raw value for each timebin were compared between the groups using a two-way ANOVA (Treatment, Time) with repeated measurements over time with treatment, period and baseline value as fixed effects and rat as random effect, completed by the following sequence:

– When the Treatment x Time interaction was significant, the Treatment effect was tested at each fixed timebin by slicing the effect according to different levels of time (SLICE command in proc MIXED). When the Treatment effect was significant for a given time-bin, a Dunnett's adjustment was performed to compare each dose to vehicle.

– When the Treatment x Time interaction was not significant, but the Treatment effect was significant, the Treatment effect was tested at each fixed timebin as an exploratory analysis by slicing the effect according to different levels of time (SLICE command in proc MIXED). When the Treatment effect was significant for a given time-bin, a Dunnett's adjustment was performed to compare each dose to vehicle.

For comparison of Vehicle versus Saline and for absolute and relative power and for each frequency band, raw value for each time bin were compared between the groups using a two-way ANOVA (Treatment, Time) with repeated measurements over time with treatment and baseline value as fixed effects and rat as random effect, completed by the following sequence:

– When the Treatment x Time interact on was significant, the Treatment effect was tested at each fixed timebin by slicing the effect according to different levels of time (SLICE command in proc MIXED).

– When the Treatment x Time interaction was not significant, but the Treatment effect was significant, the Treatment effect was tested at each fixed timebin as an exploratory analysis by slicing the effect according to different levels of time (SLICE command in proc MIXED).

Data were log-transformed before the analysis in case of departure from the assumptions of normality.

4.5 Deviations to Study Plan

During the study:

1. Rats No. 4, 7, 8, 11, and 12 were out of weight range at the time of surgery as indicated in the study plan (200-280 g). They weighed 296, 293, 304, 302 and 290 g, respectively. As there was no incidence for the surgery, the rats were included in the study.
2. Rat No. 2 was sacrificed following postoperative complications (infection at the level of the implant). This rat was included in the study and performed sessions 1 (saline) and 2 (Basmisanil). Rat No. 2 was then replaced by the rat No. 1, which performed session 1 (saline) and continued the sessions from session 3 to respect the initially planned cross-over.

3. Following the analysis of the EEG performed by CoreLab, signals of rat No. 10 were too weak in terms of power to be correctly analysed. Importantly, it was not possible to correctly discriminate wake and sleep period. As such, rat No.10 was excluded from the analysis in order not to distort the results of the study.

These deviations did not affect the integrity or validity of the results of the study.