VU0360172, a mGlu5 positive allosteric modulator, diminishes its anti-absence action after chronic ethosuximide

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ABSTRACT

Ethosuximide (ETX) has become the drug of choice in the treatment of patients with absence seizures taking into account both its efficacy, tolerability and antiepileptogenic properties. However, 47% of subjects treated with ETX failed in therapy, and most antiepileptic drugs have cognitive side effects. VU0360172, a positive allosteric modulator (PAM) of mGluR5, acutely and chronically administered decreased seizures dose dependently in rats of the WAG/Rij strain, a genetic absence model. Here it is investigated whether anti-epileptogenesis induced by ETX alters the sensitivity of VU0360172 as an anti-absence drug, and cognition is affected during and after chronic ETX treatment.

Method: Male WAG/Rij rats were chronically treated with ETX for 4 months. EEG's were recorded during and after treatment as well as challenged with VU0360172. Rats were also periodically exposed to a cue discrimination learning task in a Y-maze. mGlu5 receptors were quantified with Western Blot.

Results: Antiepileptogenesis was successfully induced by ETX and VU0360172 showed a time and dose dependent anti-absence action. However, chronic ETX treated rats showed a decrease in absences both during and after the end treatment, without clear time and dose related effects. The decrease of sensitivity for VU0360172 was not accompanied by a change in mGluR5 expression in cortex and thalamus. Chronic ETX enhanced motivation to collect sucrose pallets and this was followed by an increase in cued discrimination learning.

It is concluded that VU0360172 keeps its antiabsence effects after chronic treatment. Moreover, its differential effects in the two groups cannot be explained by a simple receptor down regulation suggesting a more downstream interaction between ETX and mGluR5. The cognitive enhancing effects of ETX, as found at the end of the experiment might be mediated to the antidepressant action of ETX as expressed by an increase in the rewarding properties of sucrose pallets.

Key words: antiepileptogenesis, mGluR5, Electroencephalography, absence epilepsy, WAG/Rij rats, Ethosuximide, Western Blots, Y-maze learning, rats

INTRODUCTION

Childhood absence epilepsy (CAE), a neurological disorder which can be found in about 10% of children with epilepsy, usually occurs around the ages of 4 to 12 years (Loiseau et al., 2002). During an absence, ongoing activity is halted and a person's responsiveness is usually briefly impaired (Panayiotopoulos, 1999). Furthermore, typical absence epileptic seizures are electrophysiologically characterized by a pattern of bilateral synchronized spike wave discharges (SWDs) (Blumenfeld et al., 2005). SWDs in the genetic rodent models such as rats of the WAG/Rij strain and GAERS are initiated in the deep layers of the somatosensory cortex and quickly spread to the cortico-thalamo-cortical (C-T-C) network (Meeren et al., 2002; Polack et al., 2007; Lüttjohann & van Luijtelaar, 2012). Current medical therapies for epilepsy symptomatically suppress seizure activity, but they are not disease modifying, having no effect on the underlying propensity of the brain to generate seizures. An exception might be the chronic treatment with ethosuximide (ETX). It might interfere with epileptogenesis as was suggested by outcomes from several experimental studies in the genetic models (Blumenfeld et al., 2008; Sarkisova et al., 2010; Russo et al., 2010). A prospective cohort study in children with CAE showed that long lasting ETX treatment resulted in a higher rate of complete remission and in more occurrences of five and ten year remission, suggestive for an anti-epileptogenic effect (Berg et al., 2014).

Evidence has emerged that metabotropic glutamate receptors (mGluR) are good candidates for the treatment of absence epilepsy (Alexander & Godwin, 2006; Doherty & Dingledine, 2002; Chapman et al., 1999). We demonstrated that the potentiation of mGlu5 receptors with the positive allosteric modulator (PAM), VU0360172, reduces SWDs dose dependently and the outcomes of a ten day chronic administration study showed no or only a very small loss of efficacy with respect to its anti-absence action (D'Amore et al., 2013; D'Amore et al., 2014). Next, it was found that VU0360172 reduces the occurrence of SWDs when locally micro-infused in the main areas of the absence network (cortex and thalamus) (D'Amore et al., 2015). This demonstrates that VU0360172 targets both locations equally effective and this may contribute to successful seizure suppression of mGluR Group I PAMs.

Considering that seizure control cannot be achieved often with monotherapy, drug interaction studies are imperative. The interaction between acutely administrated ETX and VPA showed infra-additive effects in the WAG/Rij model, suggesting that these two AED share a common

mode of action (van Rijn et al., 2004). No interactions between acutely administered ETX and non-selective orthosteric agonist/antagonist mGlu5 receptors were found in pentetrazole-induced convulsions in mice (Kłodzinskaet al., 2000; Kinga et al., 2003). However, no drug interaction studies were done in the genetic absence models during chronic ETX treatment aimed at antiepileptogenesis; it was only found that the sensitivity of levetiracetam (LEV) was lost one months after the end of its chronic treatment (Russo et al 2009). Here the (inter)action of VU0360172 with ETX will be investigated in our genetic absence model, both during and after ETX treatment has stopped.

Most AED such as VPA, ETX, and lamotrigine (LTG) have a negative impact on cognition (Conant et al., 2010; Pavone, 2001). On the other hand, the antiepileptogenic effects of chronic ETX was accompanied by a decrease in depressive-like behaviour in WAG/Rij rats (Sarkisova et al., 2010; van Luijtelaar et al., 2013). Considering that epileptogenesis and chronic ETX treatment inducing antiepileptogenesis might affect mood and cognitive processes such as learning and memory, these latter processes were additionally investigated in a cue discrimination learning task in an Y-maze (problem solving task) and in a sucrose motivation task during and after chronic treatment. This learning task has been found sensitive for the effects of e.g. VPA (Luszczki et al., 2005).
The expression of mGlu5 receptors in thalamus and cortex before and after chronic treatment with ETX and challenge with VU0360172 was additionally evaluated in order to investigate whether chronic treatment affects the expression of mGluR5 in respect to changes that could correlate with the temporal profile of drug response on SWD incidence.

Materials and Methods

Drugs and experimental protocol

ETX (Ethymal 250 mg/4 ml ethosuximide), a blocker of T-type Ca^{2+} currents in thalamic neurons (Coulter et al., 1989), was obtained from Apotex Europe BV, Leiden. VU0360172 (N-clyclobutyl-6-[2-3(fluorophenyl)ethynyl]pyridine-3-carboxamine), a selective mGlu5 receptor PAM, was obtained from Vanderbilt University Medical Center (Williams et al., 2011). ETX was administered orally through the drinking water. VU0360172 was dissolved in 10% Tween 80, and injected s.c..

Animals

The study consisted of 35 male inbred WAG/Rij rats, born and raised at the Donders Centre for Cognition in Nijmegen, The Netherlands. Nine of this rats were decapitated at one month old, none of them received ETX or vehicle. The others 26 rats, after weaning, at post natal day 28 (PND 28), were housed in standard macrolon type III cages in groups of four and five for the first two weeks, next they were housed in pairs of two. All rats had free access to food and water throughout the whole experiment, and were maintained on a 12:12 h light/dark cycle (lights on at 8.30am) under controlled conditions (20ºC, 60% humidity). The rats of this group were decapitated immediately after the last behavioural test following the last EEG recording session. All experimental procedures and animal care were carried out in compliance with the Animal Experiments Committee of Radboud University (RU-DEC). All efforts were made to minimize animal suffering and to reduce the number of animal used.

Chronic drug administration protocol and pharmacological challenge with mGluR5 PAM

For the chronic treatment studies, animals from a single litter were divided and allocated as either ETX-treated (n=13) or control (n=13) (tap water)–treated animals in a paired fashion. At 4 weeks of age, all animals were able to drink independently when the study began, ETX treatment was initiated in WAG/Rij, to achieve a palatable dose of 250 mg/kg/day in the drinking water (Blumenfeld et al., 2008, van Luijtelaar et al., 2013). Volumes consumed and animal weights were measured daily for the first week, and then weekly thereafter, and the drug dosage received (mg/kg/day) was calculated. The concentration of drug in the water bottles was updated weekly to maintain the appropriate dosage in an iterative fashion. Control-treated rats received tap water ad libitum for the duration of the study. Due to the light-sensitive nature of ETX, drinking bottles were wrapped with black tape.

Therefore, from 30 until 45 days of age, the experimental group received 150 mg ETX per 100 ml water, after which this amount was increased to 250 mg ETX per 100 ml to correspond to the amount of daily water consumption. New solutions with ETX were freshly made each week (see Fig. 1A for study timeline). To assess the effects of ETX treatment on seizures, the EEG was recorded continuously for 24h, two times: at week 16, and following drug cessation, at week 17. The drug effects on behavior were determined after 4 and 8 weeks of treatment, and at week 16 and 17 as well. The sucrose pallet motivation test was performed at 1.5 months into treatment, 4 months into treatment and 1 week after treatment had stopped.

The pharmacological challenge was scheduled with two single injections of VU0360172 (1 mg/ml, s.c. and 3 mg/ml, s.c.) at 9:00 a.m. and at 12:00 p.m. respectively in both ETX treated and control group at two different time points; during the last few days of the chronic ETX treatment and 6 days after the treatment had stopped. The EEG and the behavior of the rats was recorded (see Figure 1).

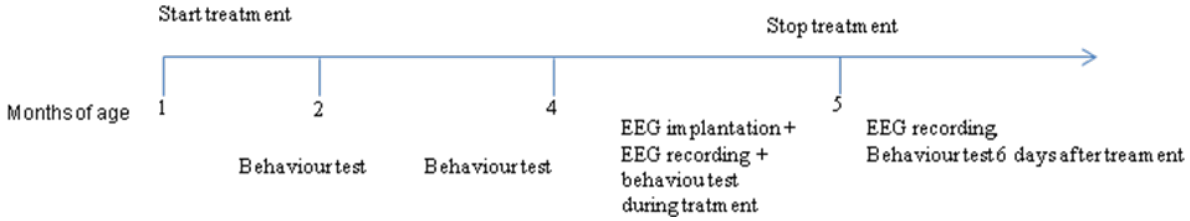


Figure.1 *Schematic diagram illustrating the experimental procedures*

In vivo recordings;

EEG Recordings

After 3 months of treatment, at the age of 4 months, the WAG/Rij rats were chronically equipped with a cortical EEG electrode set. A cortical tripolar electrode set (Plastics One®, Roanoke, VA, USA, MS333/1-A) was implanted via stereotactic surgery under isoflurane anesthesia supplemented with pre-and postoperative Rimadyl as analgesic and lidocaïne as local anesthetic.

The first electrode was implanted in the frontal region (coordinates with the skull surface flat and from bregma zero–zero, AP+2, 0: L −3, 5) with a second one in the parietal region (A −6, 0: L −4, 0) (Paxinos & Watson, 2005). The ground electrode was placed over the cerebellum. After surgery the rats had two weeks to recover, after which, they were moved into transparent EEG recording cages supplied sawdust and cage enrichment and with water and food ad libitum. WAG/Rij rats were connected to an EEG cable with a preamplifier and a swivel, which allowed free movement.

Before recording the rats were habituated to the leads for at least 24h. Each EEG session in order to check the anti-epileptogenic effect induced with ETX lasted 24 h for the treated group and control group as well. The next day after the effects of ETX during and after treatment were established, the rats were challenged with VU0360172. The EEG session for

the pharmacological challenge study with VU0360172 lasted 5 h (1h pre-injection (baseline); 2 h post the first injection; 2 h post the second injection). The differential recorded EEG was filtered (only frequencies between 1 and 100 Hz were allowed to pass) and were digitalized with a sample frequency of 512 Hz, and saved for an off-line analysis using Windaq system (DATAQ, Instruments, Akron, OH, USA). SWDs were labeled visually using common criteria, regular trains of sharp spikes and slow waves lasting from of 1–10 s, spike–wave frequency of 7–10 Hz, a spikes amplitude at least twice the background signal and asymmetric appearance of the SWDs (van Luijtelaar & Coenen, 1986; Ovchinnikov et al., 2010).

Spontaneous motor activity

Spontaneous motor activity was recorded as previously reported (van Rijn et al., 2010); an analogic passive infrared detector (PIR) (Luna PR, Rokonet Electronics LTD, Rishon Le Tzion, Israel) was fixed to a semi-open lid on top of the each rat's EEG recording cage. The analogue signal was digitalized simultaneously with the EEG signal. Movements were quantified by calculating the mean of the absolute value of the PIR signal per hour. The values of each individual rat were analyzed to investigate if there were any differences in motor activity between baseline- and post injection periods to see if there were any drug effects.

Western blot analysis of mGlu5 receptors

Nine rats were decapitated at one month of age end the other twenty-six rats at the end of the experiment and brains were rapidly removed and frozen. Brains were coded and the codes were only released after the data of the Western blot were secured. Brains were cut coronally on a cryostat, and the primary motor cortex (M1), primary somatosensory cortex (S1), reticular thalamic nucleus (RTN) and ventrobasal thalamic nuclei (VB) were manually dissected between bregma - 1.88 mm and - 3.80 mm under the guide of the Paxinos and Watson atlas (2005).

The expression of mGlu5 receptor proteins was estimated by Western blot analysis, using a highly specific polyclonal antibody (1:5000, Abcam, Cambridge, UK) and a mouse monoclonal antibody to label β-actin (1:100.000, Sigma, St. Louis, MO). Anti-mGlu5

receptor antibodies recognized a band of ~140 kDa. Specificity of anti-mGlu5 receptor antibodies was verified using brain protein extracts obtained from mGlu5 receptor knockout mice.

Brain tissues were homogenized at 4 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. After sonication, 2 μl of total extracts were used for protein determinations. One hundred micrograms of protein extract were resuspended in sodium dodecyl sulfate (SDS)-bromophenol blue reducing buffer with 40 mM dithiothreitol (DTT). Western blot analyses were carried out by loading 35 μg of total proteins per lane into 8% SDS polyacrylamide gels, which were electroblotted on immunoblot polyvinylidene difluoride (PVDF) membranes (BioRad, Milano, Italy). The PVDF membranes were blocked overnight in TBS-T buffer containing 5% non-fat dry milk. Blots were then incubated for 1 h at room temperature with rabbit polyclonal anti-mGlu5 antibodies and mouse monoclonal antibody to label β-actin. Filters were washed with TBS-T buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-rabbit or antimouse; 1:7000; Amersham, Piscataway, NJ). Immunoreactivity was revealed by enhanced chemiluminescence (ECL). Immunoreactive protein bands were quantified using the densitometry method (Scion image software, http://rsb.info.nih.gov/nihimage/). Values were obtained by calculating the ratio between the area under the curve (AUC) of the optical density of mGlu5 signal and the AUC of the house keeping protein β-actin for each lane.

Learning in the Y-maze

Problem solving has been analyzed in a Y-maze (Conrad et al., 2003; Diez-Chamizo et al., 1985; Hidaka, Suemaru, Takechi, Li, & Araki, 2011; Thompson et al., 1990). The Y-maze components were a starting box (0.10 m) in a starting arm (0.50 m) which was made with Plexiglas. Two identical arms were placed in a 90 degree angle on the starting arm and another 90 degree angle was made after 0.20 m. At the beginning of the left and right arm the cues (width 0.10 m, length 0.15 m) were presented, Plexiglas and sandpaper (Diez-Chamizo et al., 1985). The terminus of the identical arms were 0.20 m and in the termini food trays (0.03 m tall, 0.03 m diameter) were located in which sucrose pallets (Campden, sucrose pallets, 45 mg) could be placed. The Y-maze is 0.10 m in width, 0.10 m high and was covered with Plexiglas.

Procedure Y-maze

Problem solving was subsequently measured at 1.5 months into treatment, 4 months into treatment and 1 week (for 3 days) after treatment had stopped. First, rats were given a habituation period of 15 minutes 5 days before the beginning testing phase to familiarize them with the Y-maze (Conrad et al., 2003). 15 pallets were placed in the left and right arm, if the rat ate all the pallets new ones were provided. After the habituation period the rats were randomly split into two groups. The location of the cues for each rat were switched in the different testing phases (example: phase 1 left, phase 2 right, phase 3 left), the cues (whether the sandpaper was associated with food, or Plexiglas) were kept consistent over the different testing phases. The first testing phase consisted of 5 days of testing with 5 learning trials per day per rat. A learning trial took 4 minutes, the rat was placed in the closed starting box, time started running at the moment the rat left the starting box. At the side of the relevant cue 2 sucrose pallets were placed, when the rat ate the sucrose pallets (one or two) he was picked up and placed back into the starting box (Diez-Chamizo et al., 1985). The second testing phase (again 5 days and five trials per day) was the same as the first one except a time-out procedure was added after the animal made an incorrect choice. The rats were kept for 10 seconds in the arm of the incorrect cue. The third testing phase was the same as the second but only lasted 3 days. The Y-maze was cleaned after each learning trial with a 70% alcohol solution in water.
The measured variables were the percentage of correct choices and the total number of completed tasks. A task is completed by eating the sucrose pallets, next the rat was placed in the starting box again, until 4 minutes had elapsed. The criterion for making a choice was front paws and the head above/on the cue (Jones et al., 2008; Wright & Conrad, 2005).

Sucrose pallet consumption test (SPC).

The assessment of the SPC test took place in a cage exactly resembling the rat's home cage. A sucrose tray was placed against the wall of a shorter side. Rats were placed on the same location within the cage facing the sucrose tray, which contained a total of 100 sucrose pallets (Campden, 45 mg). They were allowed to explore and eat for a total of 10 minutes after which they were removed and placed back in their home cages. Rats were adapted to the food pallets 2 weeks prior to the test session and not food or water deprived. Dependent variables

measured were the number of approaches the rats made towards the sucrose tray and the total amount of sucrose pallets consumed. The SPC test was done 1.5 months of treatment, 4 months of treatment, and 1 week after the discontinuation of treatment.

Statistical analysis

All statistical procedures were performed using SPSS Version 22.0 (IBM Corp, 2013). EEG recordings lasted 5 hours starting at 9:00 a.m. for establishing the antiepileptic and antiepileptogenic effects of ETX and the vehicle group during and after one week of treatment. The behavioural and EEG part of the study were done with the two groups and the effects of chronic ETX on incidence and mean duration of SWDs, as well as the behavioural activity of animals (PIR) were tested in separate repeated-measures ANOVAs.

The incidence and mean duration of SWDs for the VU0360172 in the water group during and after treatment and for the VU0360172 challenges during and after the chronic ETX treatment was done in four separate repeated measure analysis, in an EEG time-frame of 15 -min epochs. The time of EEG recording (5 h and 10 x 15 minutes blocks post injection) was used as the within-subjects factor. In case of significant main effect (time), a simple contrast analysis was used to isolate differences across time.

Repeated measures MANOVA were also used to analyze the Y-maze data. This data set was divided across the 3 phases in which Y-maze learning occurred since there were intervals with varying length between the learning sessions. Our within-subjects factor was day (day 1 to 5, day 6 to 10, day 11-13) and the between-subjects factor was drug (ETX, control). As mentioned, the dependent variables were the percentage correct choices made in the Y-maze and the amount of completed trials.

Unpaired T-tests were used to establish differences in the SPC test, the dependent variable was the number total consumed pellets.

The experimental groups used for the Western blot were the young untreated rats, ETX treated rats, vehicle. The one-way (groups) ANOVAs, done for each brain region, were followed by post-hoc tests to establish age and treatment effects.

Results

The analysis of the incidence of SWDs in the first 5 h post injection during and after treatment showed that there was no time of day effect, or interaction with time of day with time or group (p= >.05). Instead a very large group effect was present (F= 91.955, df 3,44 , p< .000, η^2 =.862). This data confirmed the successful anti-absence and anti-epileptogenic effect induced by ETX without any difference in terms of SWD incidence between the ETX during and/or after the end of treatment. Both ETX groups showed less SWD's compared with the vehicle groups (the data are presented in Figure 2A).

No statistical difference was found in the mean duration of SWDs and in locomotor activity during and after chronic ETX treatment.

The analysis of incidence of SWD in the water group, during treatment, after the first and second injection of VU0360172 (1/3 mg/kg), revealed a large time effect (blocks) (F= 54,74, df 22,242, p< .001, η^2 = .83). A simple contrast analysis revealed that, the water group treated with VU0360172, showed clear time (the low dose of the drug was effective for about one hour, the higher dose for more than 1.5 hr) and dose dependent effects compared to the 2 hr pre-injection control period (the data are presented in Figure 2B).

The same analysis in the water group treated with VU0360172, one week after treatment showed, again a large time effects (block) (F= 57,28, df= 22,220, p<.001, η^2 =.85); again the simple contrast analysis showed time and dose (the higher dose was longer and more effective) dependent effects (Figure 2C).

The analysis of incidence of SWD in the ETX group treated with VU0360172 during treatment showed, a medium sized time effect (F= 3.07, df= 22,242, p<.001, η^2=.22). A simple contrast analysis showed that all data points indicated the same lower SWD incidence compared to the baseline value (average of 2 hours). This implies a lack of pharmacokinetic and pharmacodynamics effects as were seen in the water+VU0360172 group. The decrease in SWD incidence after VU0360172 in the ETX group suggest a kind of synergism between ETX and VU0360172 (the data are presented in Figure 2D).

Next, the same analyses on the SWD, one week after treatment, showed again a medium sized time effect (blocks) (F= 3.44, df 22,220, p< .001, η^2 = .26); again the simple contrast analysis revealed that all data points were lower if compared to the baseline value and again without clear signs of pharmacokinetic and -dynamic effects (Figure 2E).

None of the injections with VU0360172 caused significant effects between or within groups in the mean duration of SWDs and in locomotor activity.

Expression of mGlu5 receptors in cortex and thalamus of pre and symptomatic WAG/Rij rats after chronic treatment with ethosuximide.

Immunoblots showed a major band at about 140 kDa corresponding to the mGlu5 receptor monomers. The outcomes of the ANOVA showed a difference between presymptomatic (young) versus symptomatic (older) rats (F= 23.68, df 1,36, p < .001, η^2 = .397). An increased mGlu5 receptor expression was found in the cortex, and a reduced mGluR5 expression in the thalamus of old symptomatic WAG/Rij rats as compared to young rats. However, there was no significant difference in mGlu5 receptors expression in cortex and thalamus in symptomatic WAG/Rij rats after chronic treatment with ETX or vehicle (Figure 3).

Y-maze: Completed trials.

1,5 months into treatment. The univariate analysis revealed a day effect (*F*= 12.72, df 2,34, *p* < .001, η^2 = .401), a drug effect (*F*= 5.85, df 1,19, *p*> .026, η^2 = .235) and a drug x day interaction (*F*= 5.75, df 2,44, *p*> .004, η^2 = .232). The total number of completed tasks increased across days, the EXT group completed more tasks and the two groups differed in their changes across days. The ETX group were more eager to complete the task, and showed a bigger improvement over days than the control group (see Figure 4A, block 1).

4 months into treatment. The ANOVA revealed a small day effect (*F*= 3.40, df 2,34, *p* < .050, η^2 = .152) and a medial strong drug effect (*F*= 6.90, df 1,19, *p*< .017, η^2 = .266). There was no drug x day interaction. The total completed trials differed across days and the groups differed in the amount of completed tasks: the ETX group performed better than the control group (see Figure 4A, block 2).

1 week after discontinuation of treatment. The univariate analysis revealed no day effect. A drug effect was found (*F*= 13.453, df 1,19, *p*< .002, η^2 = .415): the ETX group completed more trials than the control group (see Figure 4A, block 3). There was no drug x day interaction.

Y-maze. Percentage correct.

1,5 months into treatment. The ANOVA revealed no day effect, no drug effect, and there was no drug x day interaction. Both groups performed similarly and the total average correct choices did not differ across days (see Figure 4B, block 3).

4 months into treatment. The ANOVA revealed a day effect (F= 3.692, df 3,57, p< .017, η^2 = .163). A marginal drug effect was found (F= 3.393, df 1,19, p< .081, η^2 = .152). There was no drug x day interaction. In this phase the correct choices differed across days, there is a trend indicating that the two groups differed in their correct choices but the differences between groups did not differ across days. The data showed that the ETX group performed slightly better than the control group and that the total average correct choices differed across days (see Figure 4B, block 2).

1 week after discontinuation of treatment. The ANOVA revealed a drug effect (F= 7.198, df 1,19, p< .015, η^2 = .275), no day effect and no drug x day interaction. The data showed that the ETX group performed better than the control group (see Figure 4B, block 3).

Sucrose Pallet Consumption Test (SPC).

The first SPC test has been done at 1.5 months into treatment. A large and significant effect of drug on the total consumed sucrose intake (F= 18.89, df 1,24, p< .001, η^2= .440) has been found: the ETX group consumed more pallets than the control group (see Figure 5A).

The SPC test has been done a second time, at 4 months into treatment. The data showed again a drug effect on the total consumed sucrose pallets (F= 8.41, df 1,21, p< .009, η^2= .286). The ETX group consumed more pallets than the control group (see Figure 5B).

The third SPC test has been done after treatment with ETX had stopped. The analysis revealed a marginal trend for more sucrose pallets in the ETX group (F= 3.06, df 1,19, p<.096).

Discussion

The first aim of the study was to investigate if antiepileptogenic effect induced by ETX administered via the drinking water altered the sensitivity of group I mGlu receptors. The antiabsence and antiepileptogenic effects were successfully induced by ETX without any differences in terms of SWD occurrence during the treatment and one week after the

treatment, in line with outcomes of earlier studies (Blumenfeld et al., 2008; Sarkisova et al., 2010; Russo et al., 2010, 2011; van Luijtelaar et al., 2013). Moreover, chronic ETX treatment has similar disease-modifying effects in GAERS, another genetic model of absence epilepsy (Dezsi et al., 2013). Further, some evidence showed that e.g. a non-competitive antagonist mGlu5, SIB 1893, with a pro and anticonvulsant pharmacological activity against electroconvulsive threshold-induced seizures in mice, did not influence the protective action of ETX, Valproaic acid, phenobarbital and clonazepam in this test (Kinga et al., 2003). Furthermore, combinations of antagonist mGluR5 with AED did not result in adverse effects (Borowicz et al., 2003). A lack of interaction between a selective Group II agonist and ETX and VPA on PTZ induced convulsions (Kłodzinskaet al., 2000).

Here, the sensitivity of group I mGluR in chronic ETX treated rats was evaluated by the administration of VU0360172. As expected (D'Amore et al., 2013; D'Amore et al., 2014), pharmacological enhancement of mGlu5 receptor activity caused a robust time and dose-dependent reduction with respect to the incidence of SWD in the control group. This was found two times with an interval of 6 days without obvious changes in the sensitivity of the mGlu5 receptor. However, the effects of the same doses of VU0360172 in the chronic treated ETX rats showed rather striking effects: VU0360172 decreased the incidence without any sign of time and dose-dependency. A possible explanation for the lack of dose dependency might be due to a threshold effect, it might be difficult to reduce a low number of SWDs. However, the lack of a clear pharmacokinetic effect in the ETX treated rats effect cannot be explained by a threshold effect. There was no evidence of pharmacokinetic effect in this group, the SWD incidence were slightly but significantly reduced and remained low throughout the whole recording session without returning to the baseline.

Immunoblot data of mGlu5 receptors showed a decrease in receptors expression in the thalamus between young presymptomatic versus older symptomatic rats. The decrease in the cortex was not accompanied changes in the two parts of the cortex. The thalamic changes suggest that changes in mGlu5 receptor expression are antecedent to the onset of absence seizures, and, therefore, are not secondary to seizure activity. The age related changes in the thalamus are in agreement with our earlier outcomes (D'Amore et al., 2013).

What is fascinating is that, even if the pharmacological effect of VU0360172 was completely different in the experimental and control group, the loss of dose-dependency of VU0360172 in the ETX group was not accompanied by a difference in mGlu5R expression between the two groups in cortex and thalamus. No down- or up-regulation of this receptor was found for

the chronic treated group if compared with the age matched non-treated control group, suggesting that the interaction or synergism between VU0360172 and ETX must have a different cause.

First of all, as for the ETX mechanisms, a reduction in burst-firing by ETX leaded to a reduction of SWDs by decreasing the strength of synchronization within the cortico-thalamo-cortical loop during paroxysmal activity (Leresche et al., 1998). Acute ETX, next to its well established effects on blocking T-type Ca^{2+} channels, directly reduced excitability by increasing spontaneous GABA release in cortical slices (Greenhill et al., 2012). Moreover, G protein-activated inwardly rectifying K^+ channels (GIRK (type GIRK1/2 and GIRK2)) have been shown to play an important role in regulating neuronal excitability, those channel are activated by various G_i protein-coupled receptors (Dascal, 1997; Kobayashi & Ikeda, 2006). It was found that, ETX, but not other antiepileptic drugs, at clinically relevant concentrations inhibited GIRK1/2 and GIRK2 channels in cells of cerebellar slices of mouse brain, suggesting that the inhibitory effects of ETX on GIRK channels may affect the G protein signaling pathways (Kobayashi et al., 2009). It is obvious that G-protein signaling pathways are involved in the pathophysiology of absence epilepsy and in SWD control (Alexander & Godwin, 2006; Ngomba et al., 2011). Further, the early and chronic treatment with ETX prevented the commonly reported changes in the expression of Na^+ and HCN^1 channels in the cortical focal region, as well the local excitability (Blumenfeld et al 2008; van Luijtelaar et al., 2013).

More recent research has been focused on the identification of primary cell signaling pathways that initially trigger various downstream mechanisms mediating epileptogenesis and ultimately a permanent increase in neuronal excitability (Dichter, 2006; Pitkänen & Lukasiuk, 2011). One signal transduction system that has recently gained interest as an important regulator of cellular changes involved in epileptogenesis is rapamycin (mTOR) pathway (McDaniel & Wong, 2011; Russo et al., 2012;2013; Zeng et al., 2009a). Rapamycin for 17 weeks in WAG/Rij rats had clear antiepileptogeneic effects. These chronic treatment effects might be explained by the ability of rapamycin, through mTOR inhibition, to affect a variety of cellular and molecular processes, such as ion channel expression and neurotransmitter receptor, and apoptosis, neurogenesis and synaptic plasticity all which are known to influence neuronal excitability and epileptogenesis (Tang et al., 2002; Kumar et al., 2005; Wang et al., 2006). The same kinds of both antiepileptogenic and inhibition of mTOR pathway was found also for Vigabatrin (VGB), which is known to increase in GABA levels in the brain and to

enhance SWDs in WAG/Rij rats when acutely administered (Bouwman et al., 2007), however when chronically administered, it had antiepileptogenic effects (Russo et al., 2011). VGB inhibited seizure in a mouse model and partially inhibited mTOR pathway activity and glial proliferation in mice, as well as reduced mTOR pathway activation in cultured astrocytes from mice (Bo Zhang et al., 2013). It is known that the mGluR5 antagonist activated mTOR pathway (Cao et al., 2015). It is therefore possible to speculate that the PAM VU0360172 inhibits the activity of the mTOR pathway. This suggests that both chronic ETX treatment and VU 0360172 cause an inhibition of the activity of the mTOR pathway, and this might be the reason for a diminishment of action of VU0360172 as anti-absence drug in chronic ETX treated rats, since VU0360172 might need the non-inhibited mTOR pathway to perform its anti absence action. Further study across the mTOR pathway after chronic ETX treatment might be useful in order to elucidate the involvement of mTOR signaling pathways in antiepileptogenesis.

The results from the SC test showed that WAG/Rij rats after being treated with ETX for one month are more inclined to consume sucrose pallets than untreated rats and this was replicated before the end of the chronic treatment. Although this version of sugar consumption has not been validated as a test of anhedonia (a characteristic of depression-like behaviour in WAG/Rij rats (Sarkisova et al., 2003; Sarkisova and van Luijtelaar, 2011), the outcomes do suggest an early anti-anhedonia or antidepressant-like response of ETX already at an early age and early in the treatment regime.

The Y-maze revealed also differences between both groups. Before differences in learning and memory emerged (better cue discrimination was only found at the end of treatment) the treated and untreated groups differed already in the number of completed trials after one month of ETX treatment. At this young age the treated rats seemed more motivated to run in the Y-maze and they also showed this above mentioned increased interest or appetite for sucrose pallets. Perhaps this had the consequence that these rats learned to discriminate between the cued and non-cued arm of the Y-maze. The positive effects of chronic ETX treatment on effect found was surprising, since most commonly used anti epileptic drugs such as VPA, LTG, and LEV have a negative impact on cognition (Conant et al, 2010; Chamizo-Diez et al., 1985; Pavone, 2001; Thompson et al., 1990). It is proposed that early and chronic ETX treatment has positive motivational effects and this might have facilitated the performance in a problem solving task. Whether the favorable outcomes on motivation is due

to the effects of ETX perse, or to antiepiletogenesis remains to be established. Moreover, it might be possible that the absence of early differences on percentage correct choices in the Y-maze might be explained on one hand by the duration of the treatment (too short) to induce antiepileptogenesis (van Luijtelaar et al., 2013), on the other hand that the number of trials in the learning task has been insufficient to observe an effect. The differences found in the test for problem resolution can be attributed to depressive symptoms that is suppressed in the experimental group by ETX. Further research needs to establish whether ETX is not sufficient to reduce depressive-like symptoms in humans as well, in which case additional drug treatments such as anti-depressives should perhaps be considered.

VU0360172 did not affect motor behavior, a beneficial property of a anti-absence drug. Moreover, mGlu5 receptor PAMs are already under development for the treatment of schizophrenia with the only concern of neurotoxicity and convulsive seizures induced by very high doses of these compounds (Parmentier-Batteur et al., 2013). Also the Phase II clinical trials have now demonstrated promising effects of mGluR5 NAMs in anxiety and affective disorders, Parkinsons's disease and fragile X syndrome (Emmitte, a patent review 2010-2012; Rocher et al., 2011), and suggest that the drug can be safely applied.

In conclusion, antiepileptogenesis was successfully induced by ETX, however, the chronically treated rats showed a reduction in the anti-absence action of VU0360172 both during and after chronic ETX treatment, however, synergism between ETX and VU0360172 was demonstrated. The lack of pharmacokinetic and pharmacodynamics effects remain enigmatic. The decrease of sensitivity for VU0360172 was not accompanied by a change in mGluR5 expression. The combined chronic ETX and acute VU0360172 treatment did not result in adverse effect, emphasizing the potential of mGlu receptor ligands as adjuncts to current anti-epileptic drugs.

A

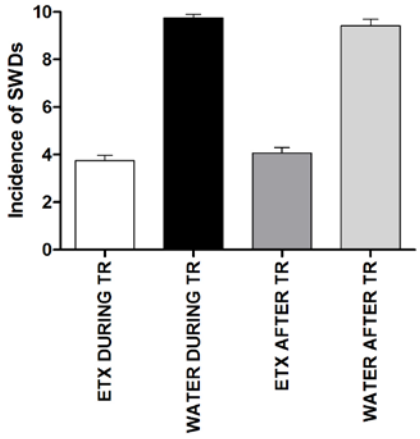
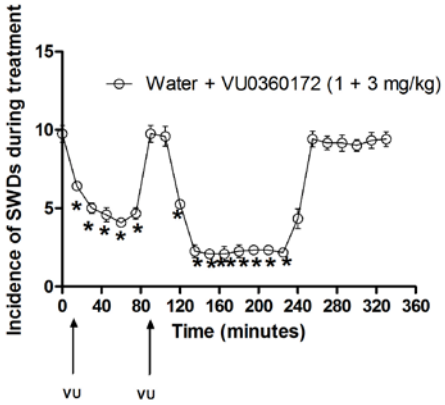
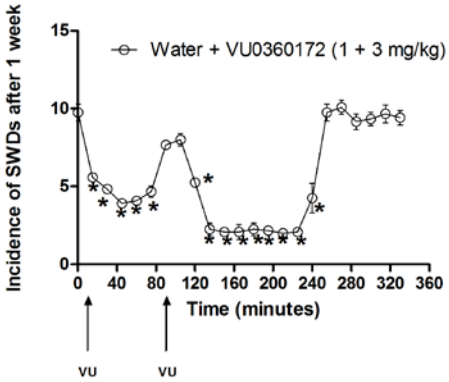


Figure 2A: Incidence of SWDs during and after ETX treatment (4 months treatment). A significant reduction of the incidence of SWDs was found during (antiepileptic) and one week after the end treatment (antiepileptogenesis).

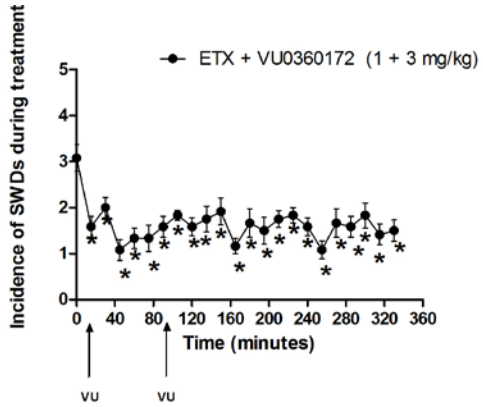
B



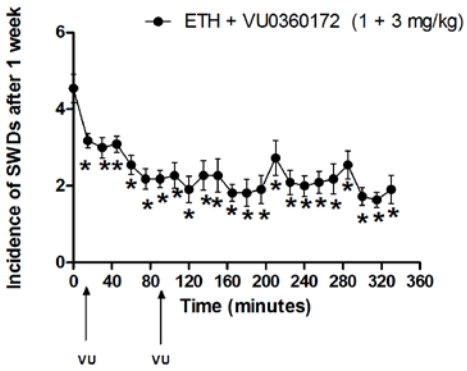
C



D



E



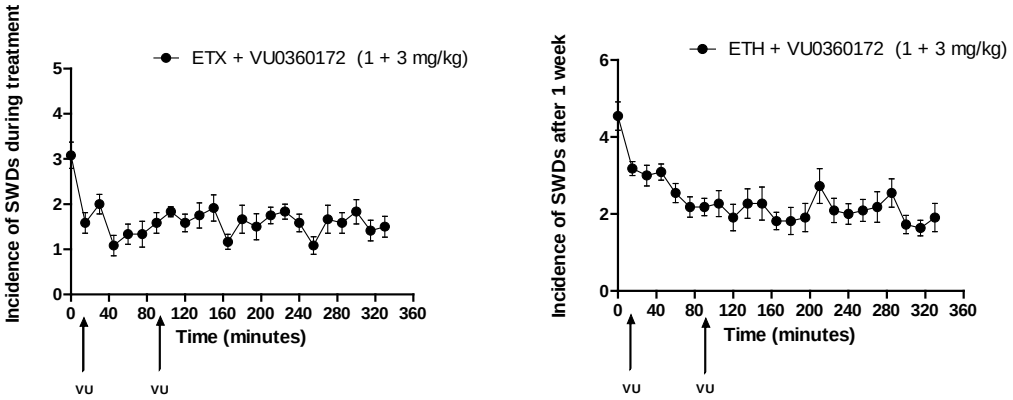


Figure 2B-C; the effect of two injections of VU0360172 in the water group during and after treatment. Notice the time and dose dependent effects of VU036172 on SWD incidence both during and treatment and after 1 week.

Figure 2D-E; the pharmacological challenge with VU0360172 (1 mg/kg and 3 mg/kg) with respect the SWD incidence during and after chronic treatment with ETX. Values are means ± S.E.M. of 22 animals. ($p < 0.05$, ANOVA for repeated measures followed by Bonferroni's test) vs. the corresponding values at baseline (T0) (*) or vs.

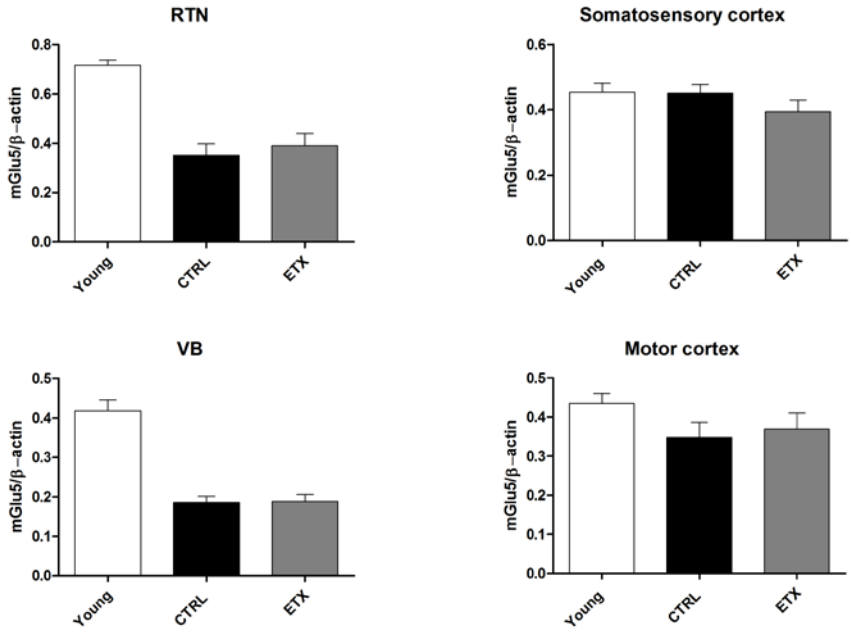


Figure 3: Mean and sem of immunoblot values of mGlu5 receptors in two parts of the thalamus (RTN, VB) and in two parts of the cortex (somatosensory and motor cortex) of young (1 month) and 5.5 month control and ETX treated WAG/Rij rats.

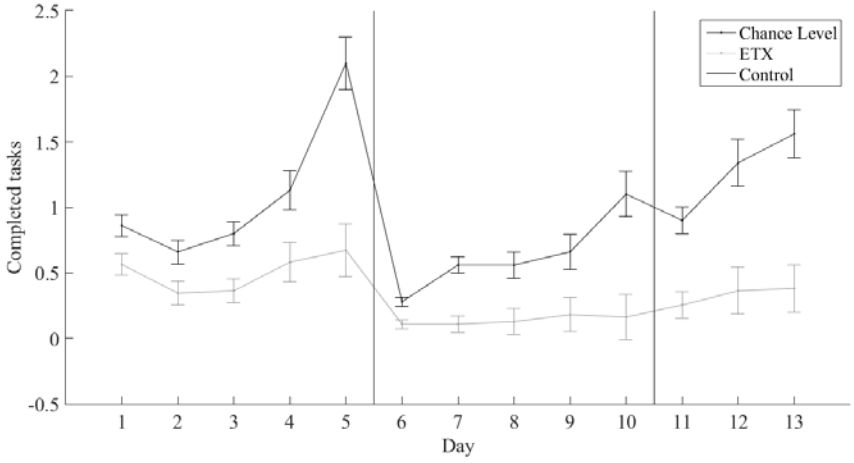


Figure 4A: Number (mean and s.e.m) of completed trials in 3 periods (day 1-5 after 1.5 month of treatment; day 6-10 after 4 month of treatment; day 11-13, 1 week after discontinuation of treatment (n = 13) and control (n = 13).

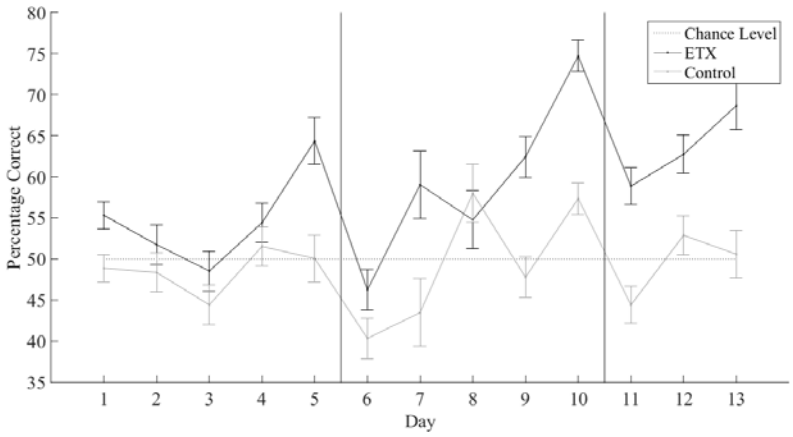


Figure 4B*:* Percentage of correct choices (mean and s.e.m) in 3 periods (day 1-5, 1.5 month of treatment, day 6-10, 4 months into treatment, day 11-13, 1 week after discontinuation of treatment), ETX (n = 13) and control (n = 13), with standard error of measurement bars.

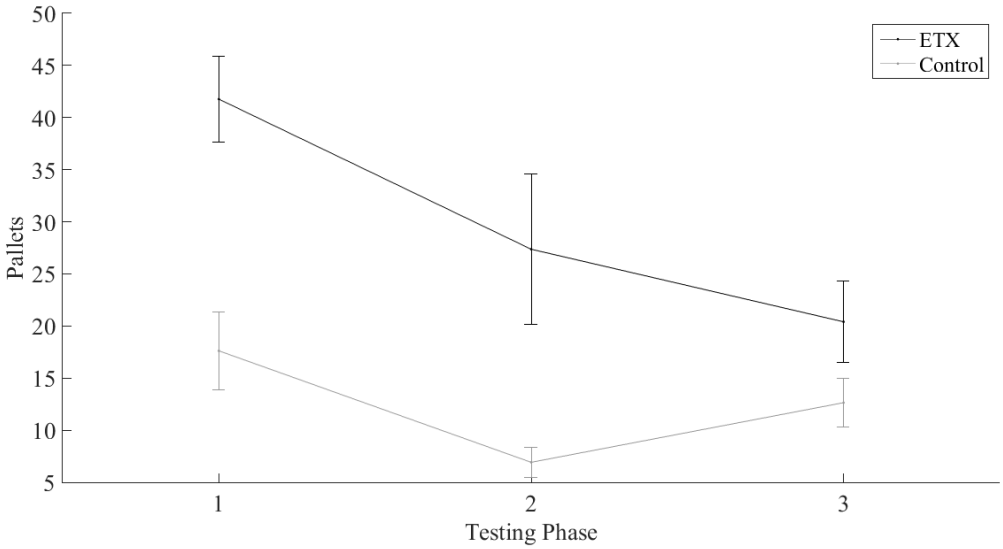


Figure 5 : Number of consumed pallets (mean and s.e.m) on 3 testing phases (1: 1.5 month of treatment, 2: 4 months into treatment, 3: 1 week after discontinuation of treatment), ETX (n = 13) and control (n = 13).

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