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**Thesis**

**Long-term effects of early-life seizures in cognitive and motor  
behaviour in mice**

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## Preface

The current thesis is part of the ESCORT (Long-term effects of early-life seizures on cortical function and excitability) project.

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## Abstract

Early-life seizures are often associated with cognitive and/or behavioural deficits. The long-term effects of single and recurrent early life seizures on behaviour were studied in mice. A single seizure was induced in 12-day old mice (P12 group) and in 22-day old mice (P22 group), while recurrent seizures were induced in a third group of mice on postnatal days 9, 11, 13 and 15 (P9-15 group) by intraperitoneal injections of pentylenetetrazole. As adults the animals underwent behavioural testing, including assessment of nest building, marble burying, open-field, elevated plus maze, social behaviour, rotarod and novel object recognition. Compared to controls, all groups of animals with seizures had reduced locomotor and exploratory activity in the open field and poorer performance in the marble burying test. Experimental animals did not differ from controls in anxiety, motor performance, recognition memory or social behaviour. These data suggest that early-life seizures in mice are mainly associated with long-term hypoactivity and low exploratory behaviour.

# 1. Introduction

## 1.1. Statement of the problem

The balance between excitation and inhibition in the cortex is critical for normal brain function and adaptive behaviour. Deviations from the excitation-inhibition balance in the cortex can lead to various degrees of hyper-excitability of the cortical network. In a state of hyperexcitability, the network can become paroxysmal causing pathological activity patterns such as epilepsy (Gutnick, Connors and Prince, 1982), while smaller degrees of cortical hyperexcitability have been associated with mental retardation syndromes (Gibson, Bartley, Hays and Huber, 2008). In the developing brain the excitation-inhibition balance is shifted in favour of excitation due to the delayed maturation of inhibitory circuits (Ben-Ari, 2006; Gaiarsa et al., 1995) - a fact that makes neonates and juveniles more susceptible to seizures (Olafsson et al., 2005). These seizures are either spontaneous or in response to a number of different insults, such as hypoxia-ischaemia, intracranial haemorrhage, birth trauma, metabolic disturbances or perinatal acquired infections (Porter, 2008; Volpe, 1973).

This developmental vulnerability becomes a clinical issue because early-life seizures are often associated with severe neurological and behavioural impairments in adult life, such as cognitive deficits (deficits in memory and learning, low academic performance, social isolation, slow psychokinetic speed) and a higher propensity for epilepsy (Bailet and Turk, 2000; Brunquell, Glennon, DiMario, Lerer and Eisenfeld, 2002; Dunn, Austin and Huster, 1997; Hughes, 2010; Reeta, Mehla and Gupta, 2010; Seidenberg et al., 1986; Sillanpaa, Jalava, Kaleva and Shinnar, 1998). In the clinic, the outcome of early-life seizures varies on an individual basis. Statistically, one third to half of these children will fare well in adulthood, while the rest will either lead a life in disease suffering from cognitive and neurological dysfunctions such as mental retardation, attention deficit disorders, behavioural disorders and epilepsy (17-40%) or will suffer premature death (16-30%) (Brunquell et al., 2002; Lombroso, 2007; Sillanpaa et al., 1998). At the same time, antiepileptic treatments can have cognitive side-effects (Reeta et al., 2010), so it is an on-going dilemma among clinicians whether to treat single or non-persistent seizures (Hughes, 2010). As a result, there is a great need to understand the mechanisms that mediate the effects of seizures *per se*, i.e. dissociated from precursor and/or concurrent underlying pathologies, and from the effects of exposure to anti-epileptic drugs.

This issue is difficult to investigate in clinical studies because there are many factors that affect the outcome, as the underlying brain pathology, age of onset, type of seizure, frequency and duration of the seizure, biological and epigenetic factors, and any pharmaceutical treatment that the patient with seizures receives. All these factors are difficult to control in human studies. In this perspective, animal studies are important because they allow for better isolation of the various contributing factors, thus fostering the study of the mechanisms that underlie the effects of seizures on brain development. Therefore, experimental models of early-life seizures are essential and rodents have been systematically used given the similarities to human seizures in terms of electrical and behavioural parameters (Kubova and Moshe, 1994). In both species, status epilepticus, the condition of persistent seizures, is manifested electrically with interictal and ictal discharges, and behaviourally with myoclonic seizures (Castro-Alamancos, 2000; Kubova and Moshe, 1994). In addition, humans and rodents have parallel behavioural profiles regarding the long-term effects of early seizures, as both develop cognitive deficits and a higher propensity for epilepsy (Holmes, Gairisa, Chevassus-Au-Louis and Ben-Ari, 1998; Sillanpaa et al., 1998). Furthermore, just like humans, young rats and mice are much more prone to seizures than adults and the consequences of seizures appear to depend on similar factors (Holmes, 1998).

## 1.2. Background

Animal studies have revealed a number of structural and/or functional effects of early-life seizures on the adult cortex, including changes in neurogenesis (Holmes, Khazipov and Ben-Ari, 2002; Porter, 2008), cell loss (Sankar et al., 2000) and synaptic reorganization (sprouting) of axons and terminals (Holmes and Ben-Ari, 1998; Holmes et al., 1998), modifications of glutamate and GABA receptors (Ni et al., 2004; Sanchez et al., 2001; Sogawa et al., 2001; Bo, Jiang, Cao, Wang and Wu, 2004; Cornejo, Mesches, Coultrap, Browning and Benke, 2007) changes in intrinsic properties (Villeneuve, Ben-Ari, Holmes and Gaiarsa, 2000) or synaptic dynamics (Isaeva, Isaev, Khazipov and Holmes, 2006; Isaeva, Isaev, Khazipov and Holmes, 2009) of cortical cells, decreases in excitatory amino acid carrier (Zhang, Raol, Hsu and Brooks-Kayal, 2004), and decreases in threshold for electrographic seizures (Isaeva, Isaev, Savrasova, Khazipov and Holmes (2010); Santos et al., 2000a). Behavioural studies have revealed changes in behaviour and cognition, reflected by deficits in learning and memory (Chang et al., 2003; Cognato et al., 2010; Holmes et al., 1998; Huang et al., 1999; Huang et al., 2002; Karnam, Zhao, Shatskikh and Holmes, 2009a;

Karnam et al., 2009b; Koh, Chung, Xia, Mahadevia and Song, 2005; Landrot, Minokoshi, Silveira, Cha and Holmes, 2001; Liu, Gatt, Werner, Mikati and Holmes, 1994; Neill et al., 1996; Nishimura, Gu, Swann, 2011; Sayin, Sutula and Stafstrom, 2004), and sensory processing (Neill et al., 1996).

Several behavioural tests have been used in order to assess any long-term cognitive or motor deficits that early-life seizures may cause. Such tests are the Morris water maze test, the open field test, the elevated plus maze test, the radial arm water maze test, the rotarod test, the inhibitory avoidance task, the Y-maze test, and the novel object (and/or novel place) recognition test.

*Spatial Learning and Memory.* The Morris water maze and radial arm water maze tests are used for short-term and long-term spatial memory and learning assessment (Morris, 1984). Most of the studies that have used these tests to evaluate the long-term effects of early-life seizures have shown that early-life seizures cause deficits in spatial learning and memory (Chang et al., 2003; Cognato et al., 2010; Holmes et al., 1998; Huang et al., 1999; Huang et al., 2002; Karnam et al., 2009a; Karnam et al., 2009b; Koh et al., 2005; Landrot et al., 2001; Liu et al., 1994; Neill et al., 1996; Nishimura et al., 2011; Sayin et al., 2004).

*Activity patterns and exploratory behaviour.* The Open field test is used for locomotor activity and exploratory behaviour assessment (Hall, 1934). Some studies have revealed that animals with early-life seizures are hypoactive and/or they show low exploratory behaviour in later life (Holmes et al., 1998; Kubova, Haugvicova, Suchomelova and Mares, 2000), while other studies have shown the opposite (hyperactivity and/or high exploratory behaviour) (Kubova et al., 2000; Santos, Arida, Filho, Priel and Cavalheiro, 2000b). The rest of the studies have not found any differences between animals with early-life seizures and animals free of seizures.

*Anxiety.* The elevated plus maze test is used for anxiety assessment (Pellow, Chopin, File, Briley, 1985). Some studies have found differences in the anxiety score between animals with early-life seizures and animals free of seizures (Kubova et al., 2004; Santos et al., 2000b; Sayin et al., 2004), while other studies have not (Cognato et al., 2010; Cornejo, Mesches and Benke, 2008; Kubova et al., 2004).

*Motor assessment.* Some studies have used the rotarod test for motor performance assessment, without finding any deficits (Huang et al., 2002; Kubova, Haugvicova, Suchomelova and Mares, 2000; Santos et al., 2000b). Other studies have used the inhibitory avoidance task and the Y-maze test for memory assessment, finding deficits in animals with

early-life seizures (Chang et al., 2003; Cognato et al., 2010; Karnam et al., 2009a; Santos et al., 2000b).

*Recognition Memory.* The novel object (and/or place) recognition test is used for recognition memory assessment. Cornejo et al. (2008) have not found any deficit in recognition memory in animals with early-life seizures.

*Experimental Induction of Seizures.* Several drugs such as flurothyl (Holmes et al., 1998; Huang et al., 1999; Karnam et al., 2009a; Karnam et al., 2009b; Landrot et al., 2001; Neill et al., 1996; Nishimura et al., 2011;), kainic acid (Cognato et al., 2010; Cornejo et al., 2008; Koh, Storey, Santos, Mian and Cole, 1999; Koh et al., 2005; Sayin et al., 2004), pilocarpine (Ikegaya, Nishiyama and Matsuki, 2000; Kubova et al., 2000; Kubova et al., 2004; Liu et al., 1994; Santos et al., 2000b), and pentylenetetrazole (Huang et al., 2002; Kornelsen, Boon, Leung and Cain, 1996) or the method of hyperthermia have been used to induce early-life seizures. Seizures are typically induced during the first 3-4 postnatal weeks between P0 and P25, they are either induced (i) once during early life (Cognato et al., 2010; Cornejo et al., 2008; Ikegaya et al., 2000; Koh et al., 1999; Koh et al., 2005; Kornelsen et al., 1996; Kubova et al., 2000; Kubova et al., 2004; Lemmens et al., 2009; Liu et al., 1994; Neill et al., 1996), (ii) every day for several days in early life (Chang et al., 2003; Huang et al., 2002; Santos et al., 2000b) or (iii) multiple times per day for several days in early life (Chang et al., 2003; Holmes et al., 1998; Huang et al., 1999; Karnam et al., 2009a; Karnam et al., 2009b; Landrot et al., 2001; Nishimura et al., 2011). Behavioural tests for the long-term effects of early-life seizures are conducted during an extended period between P24 and P100.

### 1.3. Rationale

Studying the nature of seizures during the first stages of life and whether or not they cause cognitive and/or motor deficits during adulthood is essential in order to evaluate the therapeutic value of pharmacological intervention.

Based on current literature, it is difficult to strongly conclude whether or not early-life seizures cause long-term cognitive deficits and how serious these deficits are mainly because, as mentioned above, these studies have not covered all the possible combinations of factors (e.g. time of seizures, time of behavioural tests and number of seizures). A variety of seizure induction protocols has been used in the literature. This means that several different neurological mechanisms have been exploited to induce epileptic seizures, something that

makes it even harder to group results of different studies based just on the already mentioned factors that influence the cognitive outcome of seizures. In addition, some of the drugs used for the seizure induction can have side effects such as cell loss. Furthermore, some studies do not report a complete assessment of the severity of seizures (i.e. seizure stages) and as a result they cannot be categorized as far as the seizure severity is concerned. Last but not least, current studies are often contradictory as far as their results are concerned and they do not include a wide range of behavioural tests.

As a result, there is a great need for further study organised in such a way so as to allow comparison of several factors that differentiate the long-term outcome of early-life seizures, under the same conditions.

#### 1.4. Purpose of the study

The objective of the current thesis is to test the effect of single and multiple (recurrent) early-life seizures, occurring at either the second or third postnatal week, on cognitive and motor function in adulthood. Purpose of the study is to investigate the above issues under the same conditions, taking into account as many variables as possible and using the most suitable protocols. This study investigates two different ages at which the seizures are induced, instead of one age as it is usually seen in literature. The seizures induction protocol it uses has many advantages as mentioned below and the assessment of the seizure severity is more than complete. Another purpose of the study is to cover a wide range of behavioural tests, so it includes seven different tests for the assessment of cognitive and motor function in adult animals that experienced early-life seizures. Single or multiple seizures were induced in mice during two defined developmental periods: P9-15 and P19-25, equivalent to the neonatal period and early childhood in humans, respectively (Lombroso, 2007).

The proconvulsant pentylenetetrazole (PTZ) was used to induce generalized seizures in young mice. This method has been widely used in chemically-induced animal models of generalized seizures (Kubova&Moshe, 1994). It has the advantage of being eliminated within 24 hours from the animal without any known toxic or long-term direct effects, and possesses antiepileptic sensitivity similar to humans (Loscher and Schmidt, 1988). Seizure severity was evaluated according to a well-established protocol (Luttjohann, Fabene and Luijtelaar, 2009; Pohl and Mares, 1987; Racine, 1972). Animals were brought up until adulthood, at which time the behavioural assessment was conducted.

In order to evaluate seizure-induced cognitive impairments, a battery of behavioural tests was performed in adult mice:

(i) *Nest building* and *marble burying* were conducted in order to examine species-specific spontaneous behaviour. Nest building plays a central role in the natural history of mice (nests provide them external insulation and create a less thermally stressful habitat (Clough, 1982, as mentioned by Hess et al., 2008) and therefore both male and female mice build nests spontaneously. During the nest building test mice are provided with nesting material, they are allowed to interact with it and the quality of the nests they build is then evaluated (Deacon, 2006a). *Marble burying* is genetically regulated and related to digging behaviour (Deacon, 2006b; Thomas et al., 2009), and therefore mice bury marbles spontaneously. During the marble burying test mice are allowed to explore a box covered with evenly-spaced marbles and after a specific time interval the buried marbles are counted. Poor performance in either the nest building or marble burying test, may signify a deficit in fundamental species-specific abilities and/or a propensity towards compulsive behaviour.

(ii) The *Open-field* test was conducted to examine the overall behaviour of mice, including locomotion, habituation, exploratory activity, anxiety-like behaviour and emotionality. During the open-field test, the mice are allowed to freely explore a novel environment for a defined period of time wherein several aspects of their behaviour are recorded. The total distance travelled indicates the general locomotor activity of mice, while the change in activity/locomotion with time indicates habituation. Rearing frequency (upward body placement with only hindpaws on the ground) is believed to be an index of exploratory activity, while time spent in the centre of the field assesses anxiety-like behaviour and defecation indicates emotionality (i.e. fear and/or anxiety) (Buccafusco, 2009; Choleris, Thomas, Kavaliers and Prato, 2001; Hall, 1934).

(iii) The *Elevated plus maze (EPM)* paradigm was conducted to examine anxiety-like behaviour. The elevated plus maze is an apparatus consisting of four arms (two open and two enclosed) which mice are allowed to explore freely. This test relies on the tendency of mice to prefer dark and enclosed spaces, and fear or avoid heights and open spaces. Decreased time spent in open arms/open arm entries indicates higher levels of anxiety-like behaviour. (Pellow et al., 1985; Walf and Frye, 2007).

(iv) The *Sociability test and preference for social novelty* test were conducted to examine social behaviour. Social behaviour is a critical aspect of the mouse behavioural repertoire. The sociability test was designed to assess whether experimental animals prefer a novel conspecific over an inanimate object (cage) and the social preference test was designed

to assess whether experimental animals prefer a novel conspecific over a familiar conspecific. A deviation from the expected preference for a novel conspecific in both tests is indicative of a deficit in physiological social behaviour (Moy et al., 2004; Moy et al., 2007; Moy et al., 2009).

(v) The *rotarod* test was conducted to examine motor coordination and balance. The mouse is placed on a cylinder and the speed of the cylinder rotation is gradually accelerated. Latency to fall from the rotarod is recorded. The fall is approximately 6 inches, a height that mice can easily fall and land on their feet without injury (Crawley, 1999).

(vi) The *Novel object recognition test (NORT)* was conducted to examine non-spatial learning and memory. This test includes two trials. During the first trial, mice are exposed to two identical objects (samples). During the second trial, mice are exposed to two different objects, a familiar (the sample) and a novel one. The inter-trial interval is considered as the retention period. Typically, mice discriminate between the different objects in the second trial. As they remember the previous exposure to the familiar object, they explore the novel object to a greater degree, visiting it more frequently and/or for longer periods (Ennaceur and Delacour, 1988).

## 1.5. Hypotheses

Mice that experience a single early-life seizure (at either P9-15 or P19-25), as well as mice that experience recurrent early-life seizures (at P9-15) will be compared to age-matched control mice in several behavioural aspects. It is expected that experimental animals may show possible behavioural deficits compared to control animals.

## 2. Methods

### 2.1. Overview of experiments

The animals underwent single Pentylentetrazole-induced seizures on days P12 or P22, or multiple Pentylentetrazole-induced seizures on alternate days between P9-P15. The surviving animals were left to reach adulthood (3-months old) and then underwent sequential testing in the nest building, the marble burying test, the open-field test, the elevated plus maze, the social behaviour tests, the rotarod and the novel object recognition test. The behavioural tests lasted until the mice were 102 days old. Table 1 summarizes the groups, animal numbers and tests.

Table 1. *Experimental design*

Groups (final#)	Age of injections	Nest building	Marble burying	Open field	EPM	Social behaviour	Rotarod	NORT
Group 1_exp (n=10)	P12	P88	P89	P94	P96	P97	P99	P100-102
Group 1_con (n=9)	P12	P88	P89	P94	P96	P97	P99	P100-102
Group 2_exp (n=17)	P22	P88	P89	P94	P96	P97	P99	P100-102
Group 2_con (n=9)	P22	P88	P89	P94	P96	P97	P99	P100-102
Group 3_exp (n=13)	P9-15	P88	P89	P94	P96	P97	P99	P100-102
Group 3_con (n=7)	P9-15	P88	P89	P94	P96	P97	P99	P100-102

### 2.2. Animals and housing

The study was performed in the animal facility of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens. Animal care and experimental procedures were conducted in accordance with the European Community Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes.

A total number of 81 male C57BL/6J mice were used in this study. Attempts were made to minimize the number of animals used. Male pups were chosen to eliminate the effects of hormonal cycles on behaviour. All animals were obtained from the breeding colony of the animal facility of the Foundation and were housed at a room temperature of  $24 \pm 2$  °C, a relative humidity of  $55 \pm 10\%$  and a 12h:12h light/dark cycle (07:00/19:00). Animals were maintained according to the Guide for the Care and Use of Laboratory Animals and the relevant recommendations of the European Commission on the care and use of laboratory animals. All animals had ad libitum access to water and a pelleted chow.

The day of birth was defined as postnatal day 0 (P0). Animals were housed with their litters until weaning on P28, when they were randomly divided into groups of 6-8 and housed in H-Temp™ polysulfone type III cages (365 mm (L) × 207 mm (W) × 185 mm (H), H-Temp™, Tecniplast, Buguggiate, Varese, Italy). The bedding in each cage comprised of corncob bedding (Rehofix MK 2000, J. Rettenmaier & So, Rosenberg, Germany).

### 2.3. Seizure Induction

In order to evaluate the long-term behavioural effects of early life seizures, Pentylentetrazole (PTZ; Sigma, St. Louis, MO, USA), a GABA<sub>A</sub>-receptor antagonist, was used as the seizure-inducing agent. PTZ was administered in animals after it was dissolved in saline solution (0.9% wt/vol NaCl).

The study comprised single and multiple treatments with PTZ. Under single treatment conditions, one seizure was induced on P12 (Group1) or P22 (Group2). Under multiple treatment conditions, a total of 4 seizures were induced on alternate days from P9 to P15 (Group3).

A total of 56 experimental and 25 control animals were used in the study. The control and experimental animals came from the same litters. The animals were selected randomly for the experimental or control groups.

Doses of PTZ which induce seizure of intensity stage 6, while decreasing the mortality rate were determined by pilot studies in which P12, P22 and P9-P15 animals were injected intraperitoneally with various dosages of PTZ. Group 1\_exp and Group 2\_exp animals could not be given equivalent per kilogram doses as the CD50 (convulsive dose) of PTZ increases to a peak at the animal age of 12 days and then declines to the CD50 of 8-days-old (Vernadakis and Woodbury, 1969).

Seizure intensity stages for all experimental groups were identified according to Luttjohann et al. (2009): stage 1 was characterized by sudden behavioural arrest and/or motionless staring; stage 2 by facial jerking with muzzle or muzzle and eye; stage 3 by neck jerks; stage 4 by clonic seizure in a sitting position; stage 5 by convulsions including clonic and/or tonic-clonic seizures while lying on the belly and/or pure tonic seizures and stage 6 by convulsions including clonic and/or tonic-clonic seizures while lying on the side and/or wild jumping.

*Single seizure on P12.* Experimental animals of Group 1 (Group 1\_exp, n=11) underwent a single PTZ-induced seizure on P12. Animals were injected intraperitoneally with a dose of PTZ (90mg/kg) and placed individually into clear Plexiglas cylindrical chambers (18 cm (diameter) x 20 cm (height)) for observation. All animals were continuously observed and their behaviour was at the same time recorded with a side camera for approximately 2 hours. The incidence and latency of seizure intensity stages were recorded. Onset of stage 6 occurred within 5 minutes of injection. One hour after the onset of stage 6, seizures were terminated with Diazepam (a GABA agonist, acting on GABAA receptors; Stedon, Adelco, 2 mg/kg i.p.) in order to decrease mortality (Kornelsen et al., 1996). Seizures stopped within 5 minutes of Diazepam injection and animals were allowed to recover for 40 minutes before being returned to their dams. Control animals of Group 1 (Group 1\_con, n=9) were injected intraperitoneally with equal volume of saline instead of PTZ and were separated from their dams for the same duration of time. The mothers were not observed to treat the seized pups differently than controls.

*Single seizure on P22.* Experimental animals of Group 2 (Group 2\_exp, n=30) underwent a single PTZ-induced seizure on P22. Animals were injected intraperitoneally with an initial dose of 40mg/kg PTZ, supplemented by 10 mg/kg i.p. every 15 minutes if seizure activity had not occurred. Animals were placed individually into the Plexiglas chambers and their behaviour was observed and recorded for 1-2 hours. The incidence and latency of seizure intensity stages were recorded. Onset of stage 6 occurred within 10 minutes of injection and its duration was  $13.5 \pm 1.1$  min. At the time when seizures spontaneously stopped, animals were allowed to recover for approximately 40 minutes, until they gained their baseline activity level, and then they were returned to their dams. Control animals of Group 2 (Group 2\_con, n=9) were injected intraperitoneally with equal volume of saline instead of PTZ and were separated from their dams for the same duration of time. The dams were not observed to treat the seized pups differently than controls.

*Multiple seizures on P9-P15.* Experimental animals of Group 3 (Group 3\_exp, n=15) underwent four PTZ-induced seizures in total, one on each of days P9, P11, P13 and P15. Animals were injected intraperitoneally with an initial dose of 90mg/kg PTZ, supplemented by 50 mg/kg i.p. every 15 minutes if seizure activity had not occurred. Animals were placed individually into the Plexiglas chambers and their behaviour was observed and recorded for approximately 1 hour. The incidence and latency of seizure intensity stages were recorded. Onset of stage 6 occurred within 10 minutes of injection. Fifteen minutes after the onset of stage 6, seizures were suppressed with Diazepam (Stedon, Adelco, 2 mg/kg i.p.) in order to decrease mortality. Seizures stopped within 5 minutes of Diazepam injection and animals were allowed to recover for 40 minutes before being returned to their dams. Control animals of Group 3 (Group 3\_con, n=7) were injected intraperitoneally with equal volume of saline instead of PTZ and were separated from their dams for the same duration of time. The dams were not observed to treat the seized pups differently than controls.

#### 2.4. Behavioural Testing

On P88-P102, all PTZ-treated animals that survived the seizures (N=40, in total) and their littermate saline-treated animals (N=25, in total) were assessed for various behaviour using a battery of tests. Testing was conducted in the following order: nest building (P88), marble burying (P89), open field (P94), elevated plus maze (P96), social behaviour (P97), rotarod (P99) and novel object recognition (P100-P102). Animals were handled and transferred to the behavioural testing room in order to habituate from P90 to P93. During handling, general health, body weight and neurological reflexes were also assessed.

To ensure experimenter remained blind to the treatment during the behavioural testing, all animals were numbered on P28 (the day of weaning), randomly housed into groups of 6-8, and thereafter again numbered (animal ID). During the behavioural tests, each animal was individually recognized by the animal ID number.

All the behavioural tests were done in a room with low-level illumination (30 lux), except for the marble burying test which was done with a higher level illumination (200 lux) and nest building which was conducted in the housing room during the animal dark cycle. Testing was conducted during the light phase of the animal light/dark cycle (0700–1900 h). The room temperature was always  $22\pm 1^{\circ}\text{C}$ . Animals were transferred to the behavioural testing room 1-hour before testing for acclimation period.

#### **2.4.1. Nest building**

On P88, the animals were evaluated for nest construction. Animals had no prior experience of nest building before the test. At 09:00, animals were transferred individually from their group cages to clean cages, with food, water and bedding material. At 18:30, 30 min before the dark phase, five 4×5 cm pressed white cotton squares (Pur-Zellin, Hartmann), serving as nesting material, were placed in the middle of each cage. The nesting material used for each cage was weighed in order to confirm that it was always 2g. The following morning (P89), at 09:00, nest quality was assessed. Pictures were taken and any unshredded cotton pieces were weighed. A cotton piece was considered unshredded if it weighed more than 0,1 g. Nests received a score from 0 to 5 according to a naturalistic nest scoring system of Hess et al. (2008). Score of 0 was given when there was no sign of interaction between the animal and the nesting material. Score of 1 was given when the manipulated nesting material was not gathered to a nest site. Score of 2 was given for a flat nest, characterized by gathered nesting material forming a nest cavity. Score of 3 was given for a nest with identifiable walls that form a “cup”. Score of 4 was given for a nest with walls that form an incomplete dome. Score of 5 was given for a nest with walls that completely enclose the nest hollow and form a complete dome. The nest was assigned a score by first identifying the lowest point on its edge – assigning a score of 2 through 5 – and then adding an additional 0.25 for each quarter of the nest happened to have a higher wall (Hess et al., 2008). Scoring was done by two independent observers.

#### **2.4.2. Marble burying**

Following the assessment of nest building, on P89, the animals were evaluated for marble burying. Marble burying was performed using the cages from the nest building test. Each cage was filled 3.5 cm deep with bedding material that was evened out to make a flat surface. 21 glass marbles (15 mm diameter) were gently placed on the surface of the bedding material, in a 3×7 arrangement, each 4 cm apart from the other. Each animal was placed in its cage and allowed to freely explore the cage for 30 min. Cages were covered with plastic wrap in order to avoid the animal escaping. After the 30-min period, the number of marbles buried was counted. A marble was considered as buried when at least 2/3 of the marble was covered with bedding material (Deacon, 2006b).

### **2.4.3. General health and neurological reflexes**

On P91 and P92, the animals were evaluated for general health and neurological reflexes. General health was assessed via body weight, appearance of the fur and whiskers, body posture and normality of gait. Neurological reflexes included the reactions of the eyes, the pinnae and the whiskers, to a gentle touch from a cotton swab. The animals were also observed for the ability to grasp a metal grid with forepaws and hindpaws and for the visual placing reflex (forepaw extension when slowly lowered toward a visible solid surface) (Moy et al., 2004; Moy et al., 2007; Moy et al., 2009; Vernadakis and Woodbury, 1969).

### **2.4.4. Open field**

Locomotor activity and exploratory behaviour were tested by using an open-field apparatus, consisted of a transparent Plexiglas chamber (40×40×40 cm). Testing was done between 0900 and 1700 hours, on P94. Each animal was placed in the center of the open field and allowed to explore the arena freely for 1 h. Distance traveled (cm) and rearings were measured with an overhead and side camera, respectively, using Ethovision XT8.5 specialized video tracking software. The testing chamber was cleaned between trials using ethanol 70%.

The following parameters were analyzed. (i) Locomotor activity, expressed as the total distance travelled; (ii) Habituation, expressed as the change in distance travelled during 15 minute intervals; (iii) Exploratory activity, expressed as the total number of rearings on hind paws; (iv) Anxiety-like behaviour, expressed as the time that the animal spent in the center region of the open-field area (20×20 cm); and (v) Emotionality, expressed as the total number of defecations.

### **2.4.5. Elevated Plus Maze**

On P96, the animals were assessed for anxiety-like behaviour with the elevated plus-maze. The elevated plus-maze was made of PVC and consisted of four arms 6 cm wide and 65 cm long. Two opposing arms had low walls 0,5 cm high (open arms), while the other two arms had walls 14 cm high (closed arms). The maze was elevated to a height of 50 cm above the ground. The four arms were connected by a central square (6×6 cm). Testing was done between 0900 and 1100 hours. A light source provided the same illumination to both enclosed arms. At the beginning of the experiment, each animal was placed onto the central square with its head facing an open arm and was allowed to freely explore the maze for

5 min. The maze was cleaned between animals using ethanol 70%. The following parameters were analyzed: the number of entries into open and closed arms, the time spent in open and closed arms and the total number of entries. Entry into an arm was defined as the placement of all four paws in the arm. All parameters were measured with an overhead camera, using Ethovision XT8.5 specialized video tracking software. In addition, the proportion of open-arm time was calculated for each animal. Percent open-arm time was calculated as  $100 \times (\text{time spent in open arms} / (\text{time spent in open arms} + \text{time spent in closed arms}))$ .

#### **2.4.6. Social behaviour: Sociability and Preference for social novelty tests**

The sociability testing apparatus was a rectangular, three-chambered box fabricated in house from MDF wood. Each chamber was 37cm(L)×20 cm(W)×22cm(H). Dividing walls had small square openings (10 cm × 10 cm), covered with retractable paper doorways allowing access into each chamber. Testing was done between 0900 and 1700 hours, on P97. The social behaviour testing box and the cages were cleaned with 70% ethanol between trials. The social behaviour test consisted of three 10-min sessions:

(1) *Habituation*. The test animal was placed in the middle chamber and allowed to freely explore for ten minutes. The doorways into the two side chambers were open and all the chambers were empty. Time spent in each side chamber was analyzed to ensure there was not a side preference bias for either of the two chambers of the social testing box. Time spent in each side chamber was analyzed to ensure there was not a side preference bias for either of the two chambers of the social testing box.

(2) *Sociability*. After the habituation session, the test animal was placed in the middle chamber of the social testing box and the doorways were closed. A small, round, empty wire cage (produced in house) was placed in each side chamber. The wire cages were 14.5 cm in height, with a bottom diameter of 10 cm and grid square size 1.3 x 2.5 cm. A weighted cup was embedded on the top of each cage to prevent climbing by the test mice. The cages were cleaned with 70% ethanol between trials. An unfamiliar animal (stranger 1) was placed in one of the wire cages. The location of stranger 1 in the left vs. right side chamber was systematically alternated across test animals. The doors were then opened and the test animal was allowed to freely explore the social testing box for 10 min. The test animal could choose to spend more time with an unfamiliar inanimate object with no social valence (empty cage), or with a same unfamiliar object including an unfamiliar animal (stranger 1). The following parameters were analyzed: the time spent in each chamber, the number of entries into each

chamber and the time spent sniffing each wire cage. All parameters were measured with an overhead camera, using Ethovision XT8.5 specialized video tracking software. Specifically, the parameter of sniffing was scored by two human observers, blind to animal treatment. Entry into a chamber was defined as the placement of all the animal's four limbs into that chamber. Time spent sniffing a cage was defined as time spent with the head oriented towards and within 2 cm of the cage. The reason of placing the stranger animal in a wire cage was to allow nose contact between the animals through the bars though preventing aggressive interactions, as well as to ensure that the social approach was initiated only by the test animal (Moy et al., 2007).

(3) *Preference for social novelty*. After the 10-min sociability session, the test animal was placed again in the middle chamber of the social testing box and the doorways were closed. A second unfamiliar animal (stranger2) was placed in the wire cage that had been empty during the previous session. The doors were then opened again and the test animal was allowed to freely explore the social testing box for 10 min. The test animal could prefer to spend more time with either the already investigated, familiar animal (stranger 1) or the novel unfamiliar animal (stranger 2). The time spent and the number of entries into each chamber, as well as the time spent sniffing each wire cage were calculated, in the same way as in the sociability session.

The animals used as strangers were adult male CD1 mice that had no previous physical contact with the test animals. All strangers were habituated to placement in the wire cages in the social testing box for 5 min per day, for five days before the social behaviour testing. Each stranger was used only once during the social testing. The strangers for the sociability test and the social novelty test were taken from different cages. Taking each of them from the one vs. the other cage was systematically alternated across test animals.

#### **2.4.7. Rotarod**

On P99, the animals were assessed for limb motor coordination and balance on an accelerating Rotarod (UGO BASILE). Testing was done between 1000 and 1600 hours. Animals were placed on the rotating rod with a diameter of 7 cm and they had to walk steadily forward in order not to fall from the rod. Each animal was given a habituation session and three trials to complete the test. During the 1 min habituation session, the animals were accustomed to walking on the rotating rod at the speed of 4 rpm (revolutions per minute). If the animal fell off during this session, it was placed back on the rod. Following

the habituation session, the animals were given three 5 min trials with 45 min interval between them. During each trial, the rotational speed of the rod was progressively accelerated from 4 rpm to a maximum of 40 rpm across the 5 minutes. Latency to fall was measured by the Rotarod timer. The latency value from all three trials was averaged for each animal was considered for statistical analysis. The Rotarod was cleaned with 70% ethanol between animals.

#### **2.4.8. Novel object recognition**

On P100-P102, the animals were assessed for novel object recognition, using an open-field apparatus, consisted of a Plexiglas chamber (40×40×40 cm) covered with white cardboard paper. The apparatus floor was covered with clean bedding material. Extra bedding material from each animal's home cage was added on top before the animal's testing. Testing was done between 1000 and 1600 hours. Novel object recognition task was conducted on three consecutive days.

Day 1 consisted of two 10 min trials per animal. During each trial the animal was habituated to the open field arena without objects present. Through this habituation the animal becomes familiar with the environment, and thus its interest for the objects presented during the testing day increases (Cornejo et al., 2008). The intratrial interval was 10 min, during which the animal remained in its home cage. Distance traveled (cm) and time spent in the center of the arena were measured with an overhead camera, using Ethovision XT8.5 specialized video tracking software. These data were used to assess change in locomotor activity over trials.

Day 2 also consisted of two 10 min trials per animal. During each trial the animal was placed in the open-field arena facing the centre of the opposite of the objects' side wall. The animal was exposed to two identical odourless objects that were located in a specified distance from each other (10 cm from each adjacent wall) and was allowed to freely explore. The intratrial interval was 10 min, during which the animal remained in its home cage. Time spent sniffing each object, defined as time spent with the head oriented towards and within 2 cm of the object, was analyzed for the first trial, in order to ensure there was not a side preference bias for either of the two object positions, and also to assess whether there was any tendency for neophobia by the PTZ-treated animals. The intratrial interval was 10 min, during which the animal remained in its home cage.

Day 3 consisted of one 10 min training trial followed by one 10 min testing trial per animal. In the first, training trial, the animal was exposed to the same two identical odourless objects as in the Day 2. Following the sample object exposure, the animal was returned to his home cage for a retention period of 10 min. In the second, testing trial, the animal was returned to the open-field arena and exposed to two objects in the same positions as in the training trial. The one object was used in the training trial (familiar object), while the other object was an odourless novel one (novel object) that was similar sized with the familiar. The positions of the familiar and novel objects were counterbalanced between the animals. Time spent sniffing each object was analyzed. In addition, the discrimination index was calculated for each animal, as  $100 \times (\text{time sniffing novel object} - \text{time sniffing familiar object}) / (\text{time sniffing novel object} + \text{time sniffing familiar object})$ .

All parameters were measured with an overhead camera, using Ethovision XT8.5 specialized video tracking software and scored independently by two human observers, blind to animal treatment.

## 2.5. Statistical Analysis

The statistical software PASW Statistics 18 (SPSS 18.0) was used for all statistical analyzes. The Kolmogorov–Smirnov goodness-of-fit test was used to assess normality (Gaussian-shaped distribution) for all continuous variables. Unshredded nesting material, nest score, marbles buried, distance travelled, total entries (EPM), percent open-arm time (EPM) and latency to fall off the rotarod, were statistically evaluated using Student's t-test comparisons between animals that received PTZ or animals that received saline. Sociability and social novelty preference were evaluated using two-way repeated measures ANOVA, with treatment (PTZ, saline) as between factor and chamber side (e.g. stranger 1, stranger 2) as within factor. Preference for open vs closed arms in elevated plus maze was evaluated using two-way repeated measures ANOVA, with treatment as between factor and arms as within factor. Recognition memory in novel object recognition test was evaluated using two-way repeated measures ANOVA, with treatment as between factor and object as within factor. Distance travelled during quarters in open field test was evaluated using two-way repeated measures ANOVA with a Bonferroni post hoc test to correct for multiple comparisons. The correlation between marbles buried and the distance traveled during that

test was evaluated using Pearson correlation coefficient. Results are presented as means  $\pm$  standard errors. For all comparisons, statistical significance was set at  $p < 0.05$ .

### 3. Results

#### 3.1. Behavioural features of PTZ-induced seizures

All animals in the experimental groups had seizures induced by PTZ. The behavioural expressions of seizures sometimes consisted of sudden behavioural arrest (stage 1, according to Luttjohann et al., 2009), facial jerking (stage 2, according to Luttjohann et al., 2009) and neck jerks (stage 3, according to Luttjohann et al., 2009), but always consisted of clonic seizures in a sitting position (stage 4, according to Luttjohann et al., 2009), clonic and tonic-clonic seizures while lying on the belly and pure tonic seizures (stage 5, according to Luttjohann et al., 2009) and clonic and tonic-clonic seizures while lying on the side and wild jumping (stage 6, according to Luttjohann et al., 2009). Clonic seizures were manifested by repetitive, rapid convulsions of forelimbs and hindlimbs, with a loss of the righting reflex. Tonic seizures were manifested by full extension of forelimbs and hindlimbs.

Mortality rate was 9,1% for the Group 1\_exp (single seizure on P12), 43,3% for the Group 2\_exp (single seizure on P22) and 13,3% for the Group 3\_exp (multiple seizures on P9-15). None of the saline-treated animals died.

#### 3.2. Behavioural Testing

None of the animals in either group presented behavioural seizures during the testing periods.

##### **3.2.1. General health and neurological reflexes**

All animals displayed normal general health and normal neurological reflexes in response to tactile and visual stimuli. All animals displayed normal vision on the visual placing reflex, although one animal belonging to the Group 3\_exp group was excluded from the behavioural testing as it had one eye half-open. At the beginning of testing the animals had body weights in the range of 26–28 grams.

##### **3.2.2. Nest building**

###### *Animals that survived single seizures on P12*

The nests of PTZ-treated animals did not differ from those of saline-treated. Student's t-test showed no difference in the percent of nesting material left unshredded between the PTZ-treated (N=10) and saline-treated (N=9) animals [ $t(17) = -0.69, p = .498$ ].

No difference was found between PTZ-treated (N=10) and saline-treated (N=9) animals in nest score [ $t(17) = 1.08, p = .297$ ].

*Animals that survived single seizure on P22*

There was no difference in the percent of nesting material left unshredded between the PTZ-treated (N=17) and saline-treated (N=9) animals [ $t(24) = 0.08, p = .933$ ].

No difference was found between PTZ-treated (N=16) and saline-treated (N=9) animals in nest score [ $t(10.13) = 1.31, p = .221$ ].

*Animals that survived multiple seizures on P9-P15*

Student's t-test showed no difference in the unshredded nesting material between the PTZ-treated (N=12) and saline-treated (N=6) animals [ $t(16) = -0.54, p = .595$ ].

No difference was found between PTZ-treated (N=12) and saline-treated (N=7) animals in nest score [ $t(9.06) = -1.89, p = .090$ ].

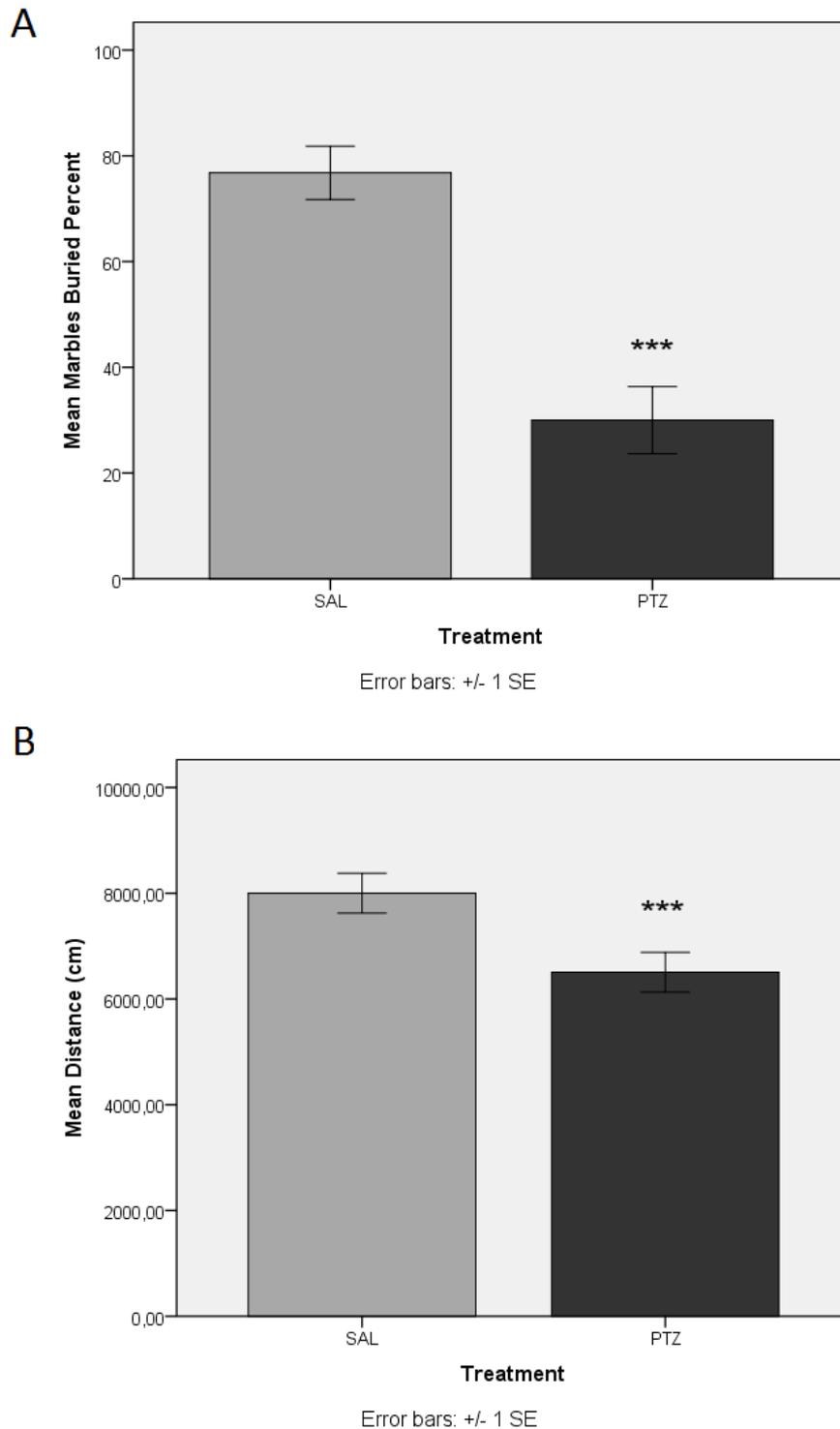
### **3.2.3. Marble burying**

*Animals that survived single seizure on P12*

PTZ-treated animals (N=10) buried significantly fewer marbles than saline-treated animals (N=8) [ $t(16) = 5.55, p < .001$ ] (Fig. 1A).

The distance travelled during the 30-min trials was also measured. PTZ-treated animals (N=10) were less active than saline-treated (N=9) during marble burying [ $t(17) = 4.99, p < .001$ ] (Fig. 1B).

Pearson's correlation analysis revealed that percent marbles buried was significantly and highly correlated with the distance travelled during the marble burying test [ $r(18) = .77, p < .001$ ], suggesting that the reduced marble burying may have been secondary to reduced locomotion.



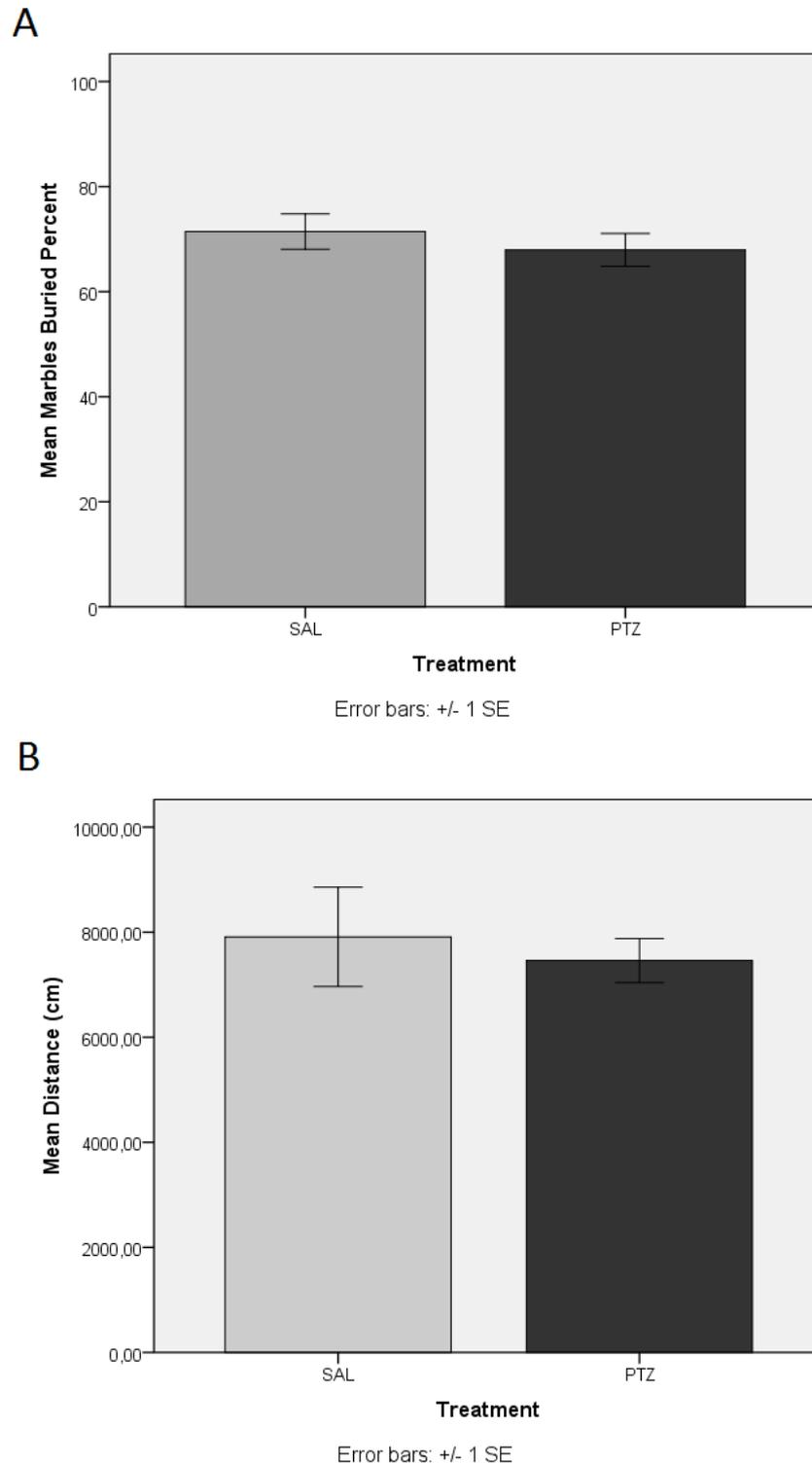
*Figure 1.* (A) Comparison of marble burying performance between the animals with a single seizure on P12 (PTZ-treated) and controls (saline-treated). Animals with early life seizure buried significantly fewer marbles than controls ( $p < .001$ ). (B) Comparison of distance travelled during the marble burying test between the animals with early life seizures and controls. Animals with early life seizures were less active than controls ( $p < .001$ ).

*Animals that survived single seizure on P22*

There was no significant difference in marble burying between PTZ-treated (N=15) and saline-treated (N=5) animals [ $t(18) = 0.6, p = .556$ ] (Fig. 2A).

The distance travelled during the 30-min trials was also measured. Student's t-test showed that PTZ-treated animals (N=13) were as active as saline-treated (N=5) during this test [ $t(16) = 0.51, p = .617$ ] (Fig. 2B).

Pearson's correlation analysis revealed that the percent marbles buried was significantly low correlated with the distance travelled during the marble burying test [ $r(17) = 0.49, p < .05$ ].



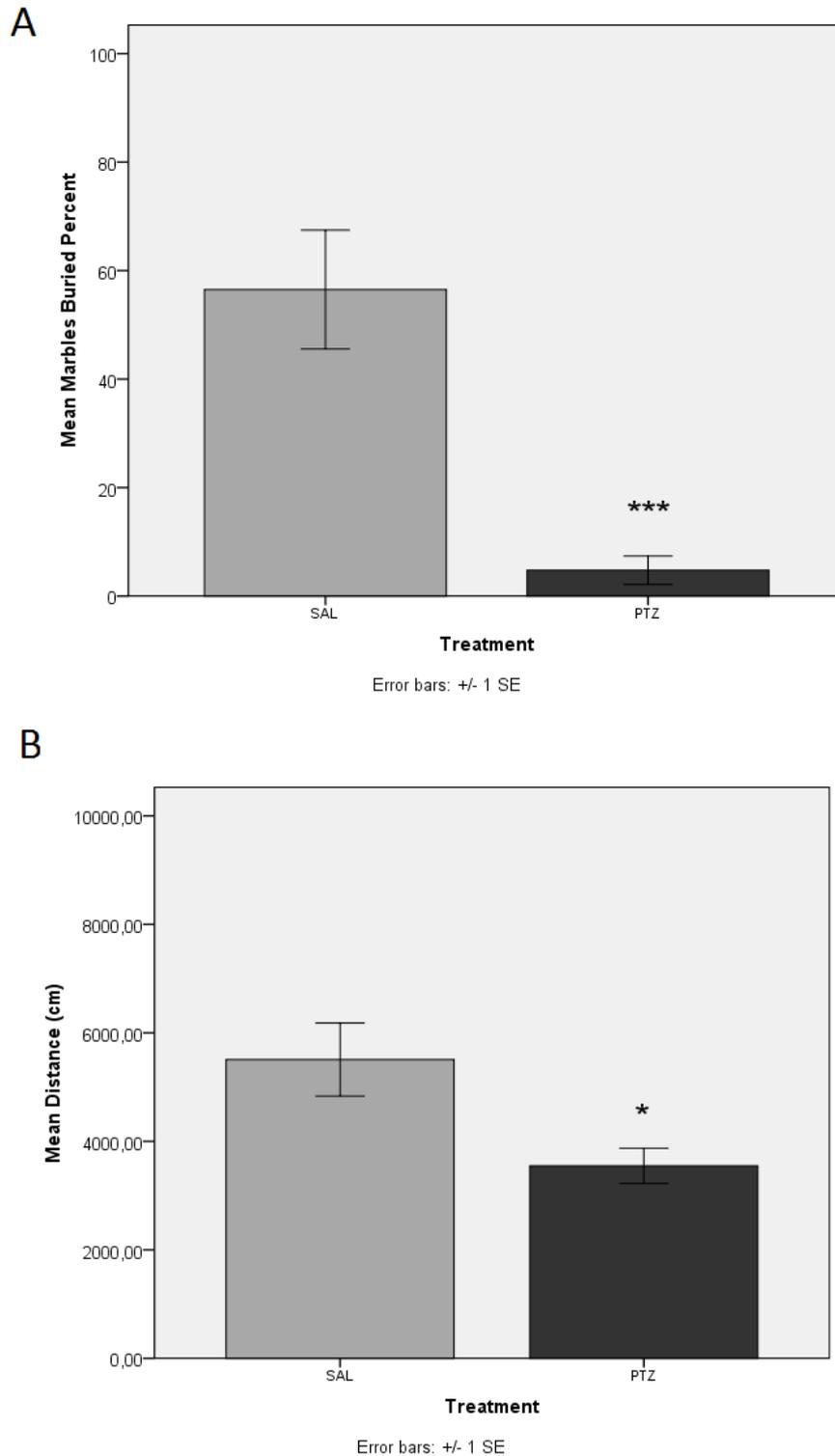
*Figure 2.* (A) Comparison of marble burying performance between the animals with a single seizure on P22 (PTZ-treated) and controls (saline-treated). No difference was found between animals with early life seizure and controls ( $p=.556$ ). (B) Comparison of distance travelled during the marble burying test between the animals with early life seizures and controls. No difference was found between animals with early life seizures and controls ( $p=.617$ ).

*Animals that survived multiple seizures on P9-P15*

PTZ-treated animals (N=11) buried significantly less marbles than saline-treated (N=7) [ $t(16) = 5.51, p < .001$ ] (Fig. 3A).

The distance travelled during the 30-min trials was also measured. Student's t-test showed that PTZ-treated animals (N=11) were less active than saline-treated (N=7) during this test [ $t(16) = 2.93, p = .010$ ] (Fig. 3B).

Pearson's correlation analysis revealed that the marbles buried percent significantly and highly correlated with the distance travelled during the marble burying test [ $r(17) = .73, p < .001$ ].



*Figure 3.* (A) Comparison of marble burying performance between the animals with multiple seizures on P9-15 (PTZ-treated) and controls (saline-treated), Animals with early life seizures buried significantly less marbles than controls ( $p < .001$ ). (B) Comparison of distance travelled during the marble burying test between the animals with early life seizures and controls. Animals with early life seizures were less active than controls ( $p < .05$ ).

### 3.2.4. Open field

#### *Animals that survived single seizure on P12*

PTZ-treated animals (N=9) were less active than saline-treated animals (N=7). There was a significant effect of treatment [ $t(10.4) = 3.99, p < .01$ ] on total distance travelled during the 1-hour open field test (Fig. 4A).

Examining the distance travelled during the four 15 minute periods (quarters) of the one hour open field test (Fig. 4B), two way repeated measures ANOVA with post hoc analysis showed no interaction of treatment $\times$ quarter [ $F(3, 42) = 0.23, p = .877, \eta^2 = .016$ ]. There was a significant main effect of treatment [ $F(1, 14) = 12.91, p < .01, \eta^2 = .480$ ], indicating that during all four quarters the PTZ-treated animals had significantly lower levels of distance travelled than the saline-treated animals. There was also a significant effect of quarter [ $F(3, 42) = 51.14, p < .001, \eta^2 = .785$ ], indicating that all animals were habituated to the open-field environment. Multiple comparisons (Bonferonni correction) showed that the ambulatory distance for both PTZ-treated and saline-treated animals was significantly reduced during all quarters compared with the previous ones ( $p < .001$  for the first quarter compared with the second, third and fourth, and for the second quarter compared with the fourth;  $p < .05$  for the third quarter compared with the fourth), except for the second quarter compared with the third ( $p = .077$ ).

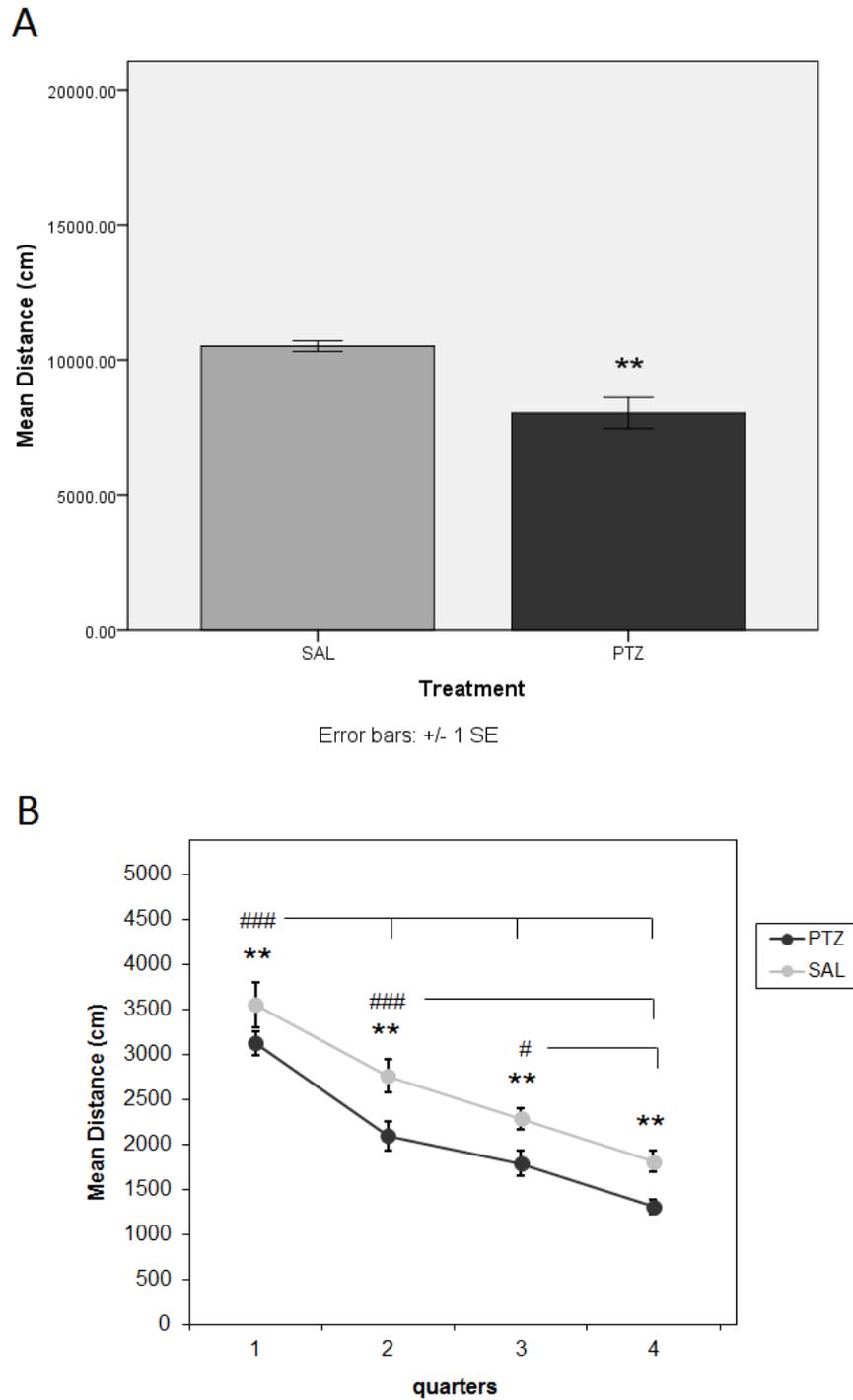
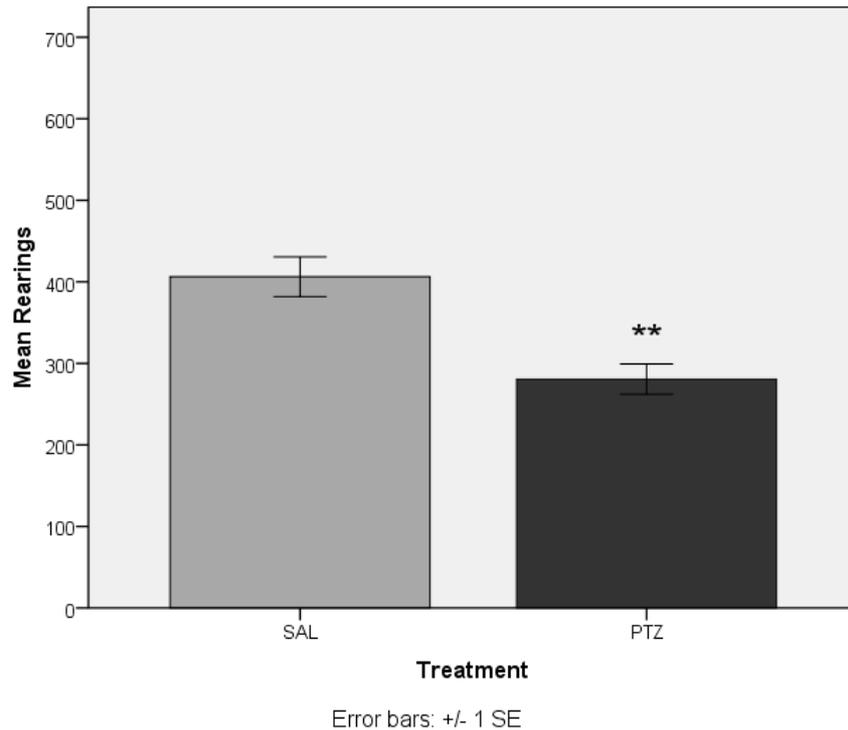


Figure 4. (A) Comparison of total distance travelled during the open-field test between the animals with a single seizure on P12 (PTZ-treated) and controls (saline-treated). Animals with early life seizures were less active than controls ( $p < .01$ ). (B) Distance travelled during the four quarters of the one hour open field test. All animals exhibit habituation to the open-field environment.

There was a significant difference in exploratory activity, with PTZ-treated animals (N=9) performing fewer rearings than saline-treated animals (N=10) during the one hour open field test [ $t(17) = 4.16, p = .001$ ] (Fig. 5). Examining the number of rearings during each of the four 15 minute periods (quarters), two way repeated measures ANOVA with post hoc analysis showed no interaction of quarter  $\times$  treatment [ $F(3, 39) = 1.34, p = .276, \eta^2 = .093$ ]. As in the total distance measure, there was a significant main effect of treatment [ $F(1, 13) = 44.87, p < .001, \eta^2 = .775$ ], indicating that during all four quarters the PTZ-treated animals had significantly lower levels of rearings than the saline-treated animals. There was also a significant effect of quarter [ $F(3, 39) = 10.93, p < .001, \eta^2 = .457$ ]. Multiple comparisons (Bonferonni correction) showed that there was a significant decrease in the number of rearings for both PTZ-treated and saline-treated animals from the first quarter to the third and fourth ( $p < .01$ ) and from the second to the fourth ( $p < .05$ ).

There were no differences between PTZ-treated and saline-treated animals in the total time spent in the center region of the open field [ $t(15) = 0.79, p = .441$ ] or the number of defecations [ $t(17) = -0.15, p = .88$ ].



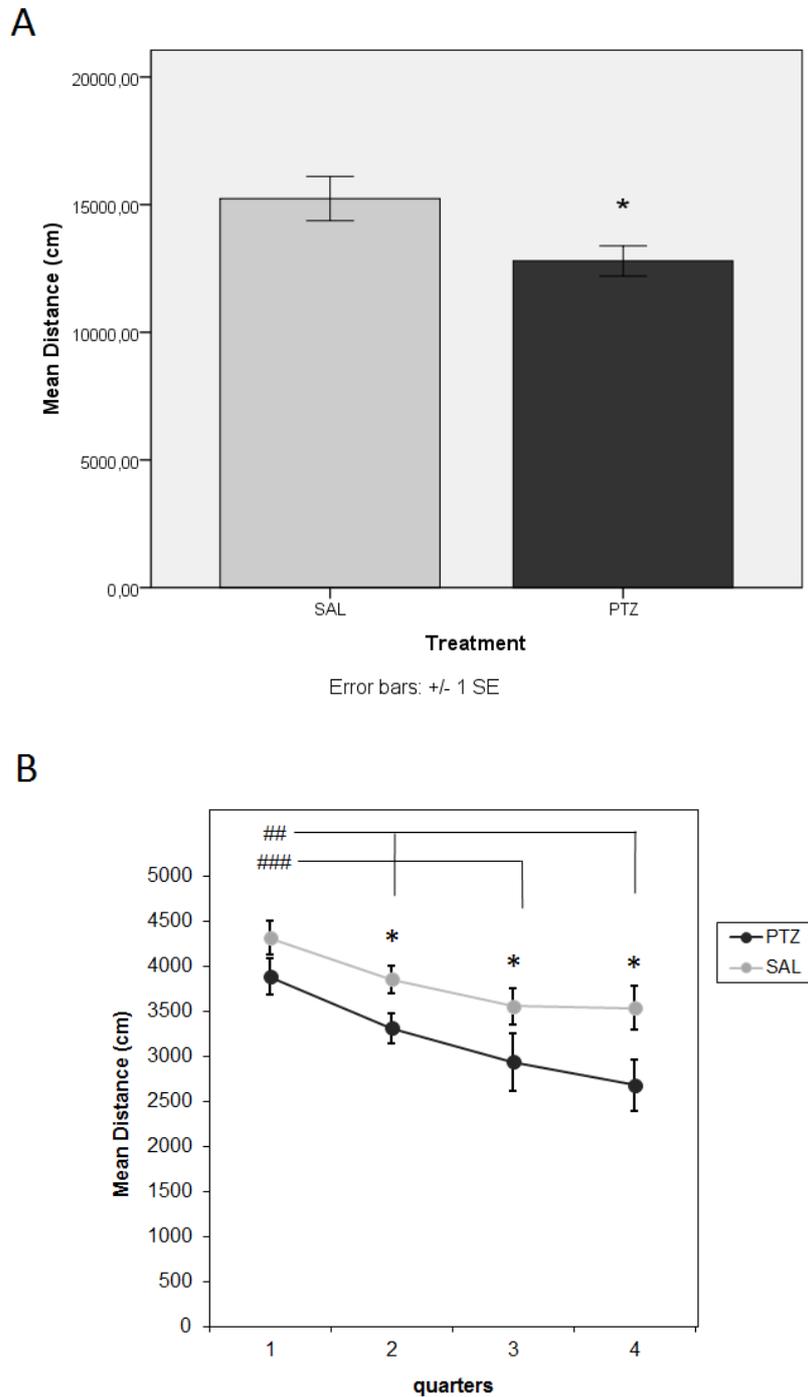
*Figure 5.* Comparison of total rears during the open-field test between the animals with a single seizure on P12 (PTZ-treated) and controls (saline-treated). Animals with early life seizures were less explorative than controls ( $p < .01$ ).

#### *Animals that survived single seizure on P22*

There was a significant difference in locomotor activity between PTZ-treated ( $N=16$ ) and saline-treated ( $N=8$ ) animals. A significant effect of treatment was found on total distance travelled during the open field test [ $t(22) = 2.36, p < .05$ ] (Fig. 6A).

Examining the distance travelled during the four quarters of the one hour open field test (Fig. 6B), two way repeated measures ANOVA with post hoc analysis showed no interaction of treatment  $\times$  quarter [ $F(3, 66) = 0.55, p = .651, \eta^2 = .024$ ]. There was a significant main effect of treatment [ $F(1, 22) = 5.57, p < .05, \eta^2 = .202$ ], demonstrating that during all four quarters the PTZ-treated animals had significantly lower levels of distance travelled than the saline-treated animals. There was also a significant effect of quarter [ $F(3, 66) = 13.02, p < .001, \eta^2 = .372$ ], indicating that all animals were habituated to the open-field environment. Multiple comparisons (Bonferonni correction) showed that there was a significant decrease in ambulatory distance for both PTZ-treated and saline-treated animals from the first 15-minutes to the second ( $p < 0.01$ ), third ( $p < 0.001$ ) and fourth ( $p < 0.01$ ) ones. The decrease from the

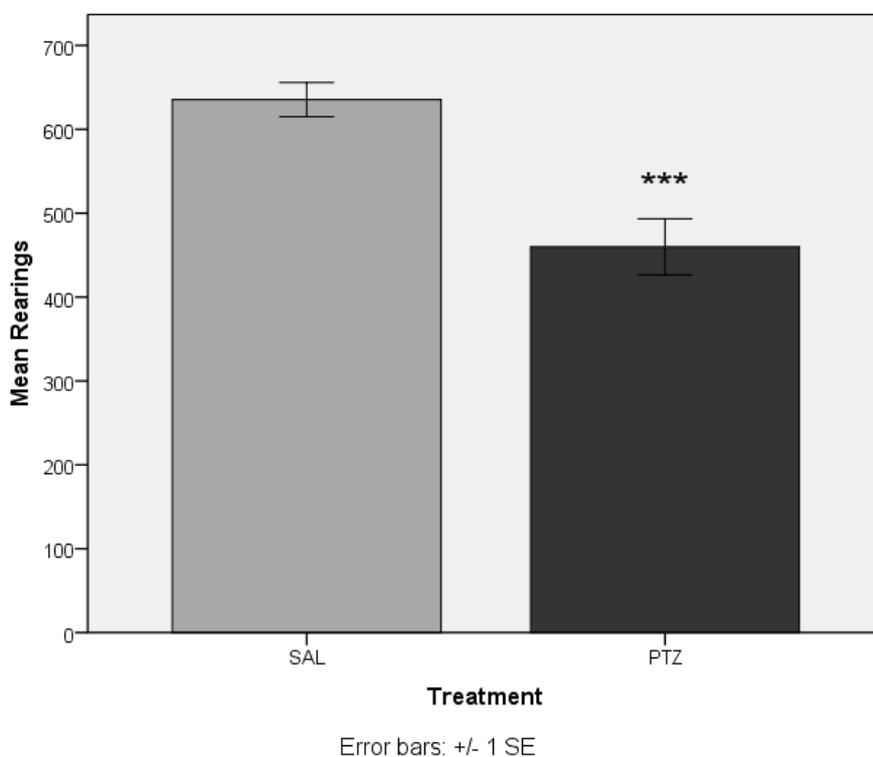
second quarter to the third and fourth ones was not significant ( $p = .251$  and  $p = .099$ , respectively), neither it was from the third to fourth quarter ( $p = 1$ ).



*Figure 6.* (A) Comparison of total distance travelled during the open-field test between the animals with a single seizure on P22 (PTZ-treated) and controls (saline-treated). Animals with early life seizures were less active than controls ( $p < .05$ ). (B) Distance travelled during the four quarters of the one hour open field test. All animals showed habituation to the open-field environment.

There was a significant difference in exploratory activity, with PTZ-treated animals (N=17) manifesting fewer rearings than saline-treated animals (N=8) [ $t(22.9) = 4.47$ ,  $p < .001$ ] (Fig. 7). Examining the number of rearings during the four 15 minute periods (quarters) of the one hour open field test, two way repeated measures ANOVA with post hoc analysis showed no interaction of quarter  $\times$  treatment [ $F(3, 72) = 1.72$ ,  $p = .170$ ,  $\eta^2 = .067$ ]. There was a significant main effect of treatment [ $F(1, 24) = 7.21$ ,  $p < .05$ ,  $\eta^2 = .231$ ], indicating that during all four quarters the PTZ-treated animals had significantly lower levels of rearings than the saline-treated animals. There was also a significant effect of quarter [ $F(3, 72) = 6.05$ ,  $p < .01$ ,  $\eta^2 = .201$ ]. Multiple comparisons (Bonferonni correction) showed that there was a significant increase in the number of rearings for both PTZ-treated and saline-treated animals only from the first quarter to the second one ( $p < .001$ ).

There were no differences between PTZ-treated and saline-treated animals in the total time spent in the center region of the open field [ $t(21) = -0.02$ ,  $p = .983$ ] or the number of defecations [ $t(23) = -0.67$ ,  $p = .948$ ].



*Figure 7.* Comparison of total rears during the open-field test between the animals with a single seizure on P22 (PTZ-treated) and controls (saline-treated). Animals with early life seizures were less explorative than controls ( $p < .001$ ).

*Animals that survived multiple seizures on P9-P15*

PTZ-treated animals (N=10) were less active than saline-treated (N=7). There was a significant effect of treatment on total distance travelled during the open field test [ $t(15) = 4.9, p < .001$ ] (Fig. 8A).

Examining the distance travelled during the four quarters of the one hour open field test (Fig. 8B), two way repeated measures ANOVA with post hoc analysis showed no interaction of treatment $\times$ quarter [ $F(3, 45) = 0.44, p = .724, \eta^2 = .029$ ]. There was a significant main effect of treatment [ $F(1, 15) = 24.08, p < .001, \eta^2 = .616$ ], indicating that during all four quarters the PTZ-treated animals had significantly lower levels of ambulatory distance than the saline-treated animals. There was also a significant effect of quarter [ $F(3, 45) = 26.88, p < .001, \eta^2 = .642$ ]. Multiple comparisons (Bonferonni correction) showed that the ambulatory distance for both PTZ-treated and saline-treated animals was significantly reduced during all quarters compared with the previous ones ( $p < .01$  for the first quarter compared with the second and third, and for the fourth quarter compared with the second and third ;  $p < .001$  for the first compared with the fourth) except for the second 15-minutes compared with the third ( $p = .38$ ).

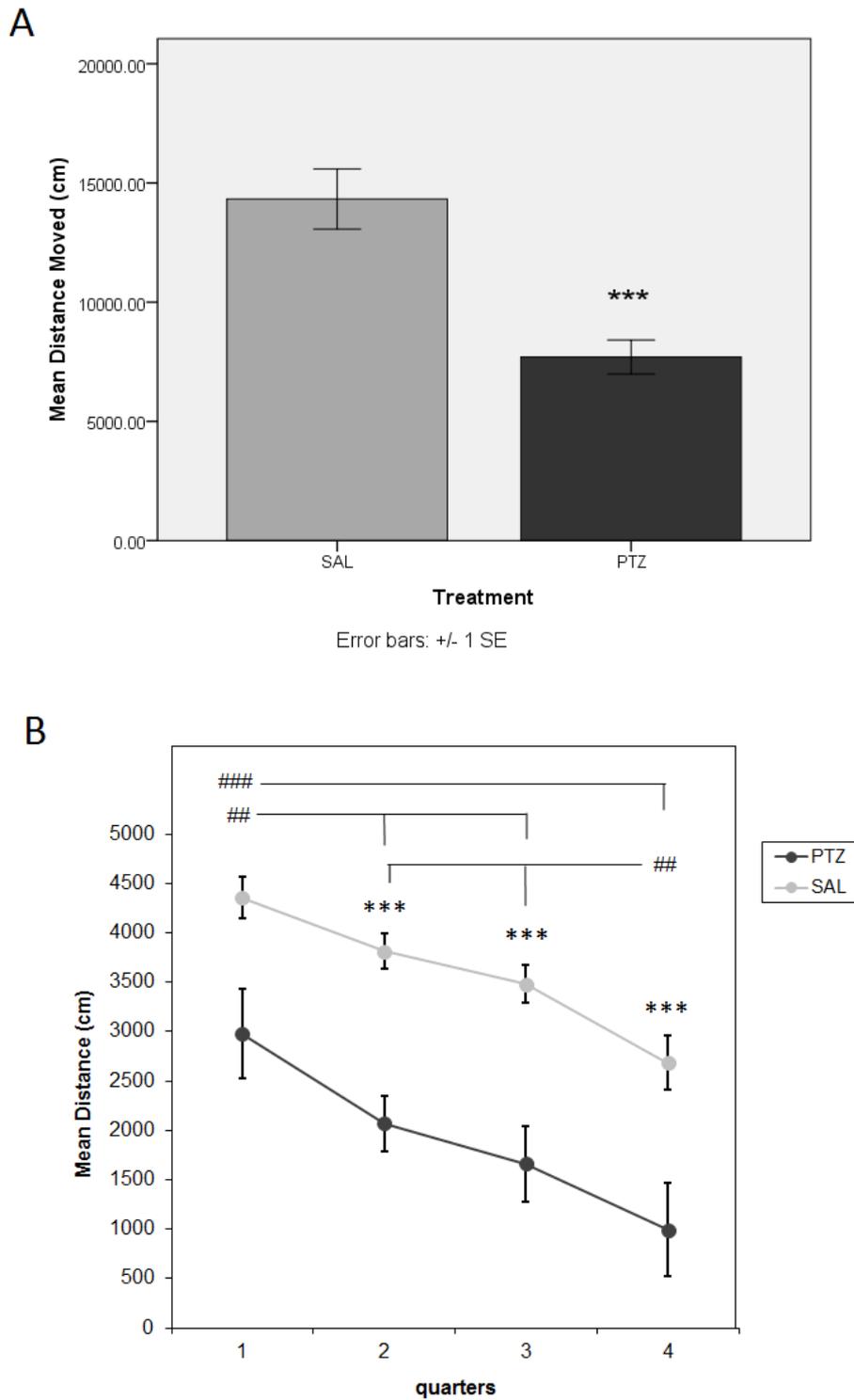
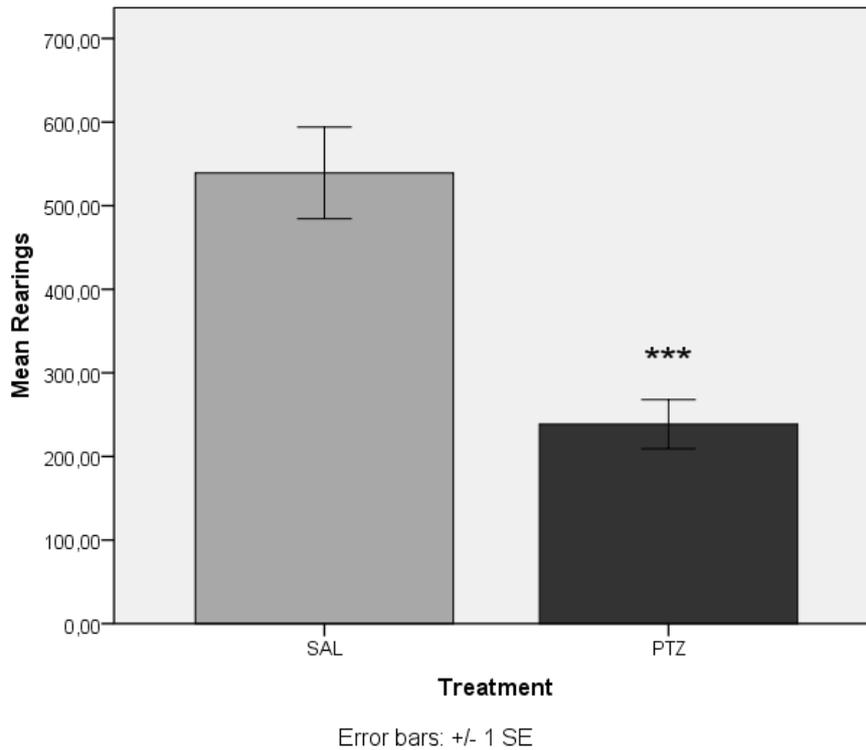


Figure 8. Comparison of total distance travelled during the open-field test between the animals with multiple seizures on P9-15 (PTZ-treated) and controls (saline-treated). Animals with early life seizures were less active than controls ( $p < .001$ ). (B) Distance travelled during the four quarters of the one hour open field test. All animals showed habituation to the open-field environment.

There was a significant difference in exploratory activity, with PTZ-treated animals (N=10) manifesting fewer rearings than saline-treated animals (N=6) [ $t(14) = 5.32, p < .001$ ] (Fig. 9). Examining the number of rearings during the four 15 minute periods (quarters) of the one hour open field test, two way repeated measures ANOVA with post hoc analysis showed no interaction of quarter  $\times$  treatment [ $F(3, 45) = 0.51, p = .675, \eta^2 = .033$ ]. There was a significant main effect of treatment [ $F(1, 15) = 15.00, p < .01, \eta^2 = .500$ ], indicating that during all four quarters the PTZ-treated animals had significantly lower levels of rearings than the saline-treated animals. There was also a significant effect of quarter [ $F(3, 45) = 6.07, p = .001, \eta^2 = .288$ ]. Multiple comparisons (Bonferonni correction) showed that there was a significant increase in the number of rearings for both PTZ-treated and saline-treated animals from the first quarter to the second one ( $p < .05$ ), and a significant decrease from the second to the fourth quarter ( $p < .05$ ) and from the third to the fourth ( $p < .01$ ).

There were no differences between PTZ-treated and saline-treated animals in the total time spent in the center region of the open field [ $t(15) = 0.36, p = .723$ ] or the number of defecations [ $t(17) = 1.04, p = .312$ ].

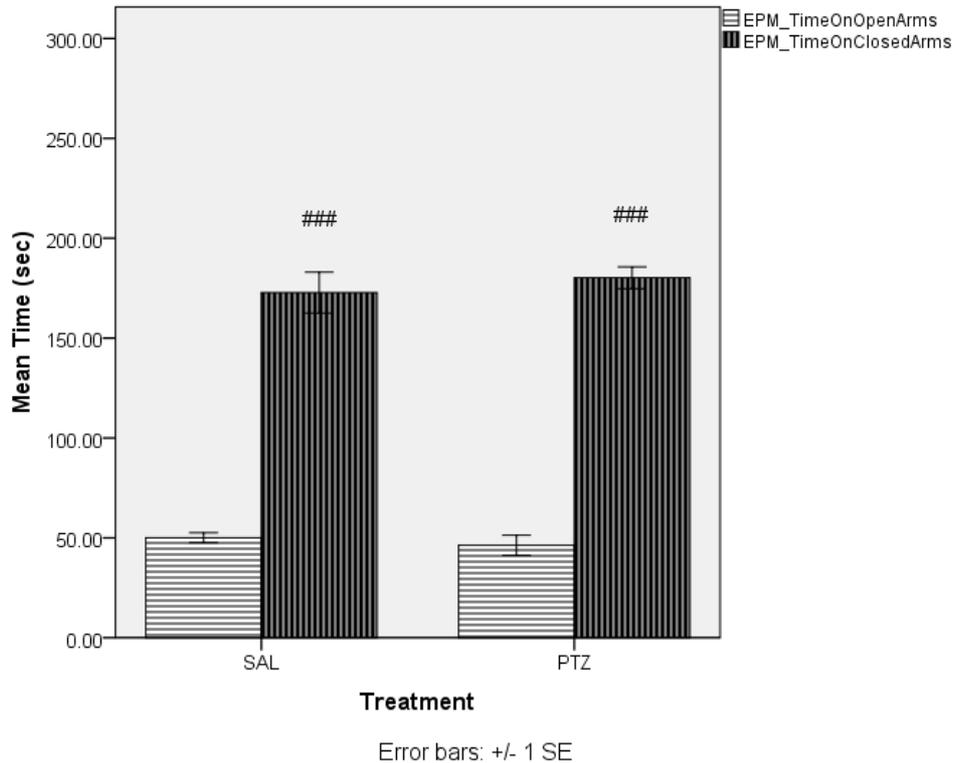


*Figure 9.* Comparison of total rears during the open-field test between the animals with multiple seizures on P9-15 (PTZ-treated) and controls (saline-treated). Animals with early life seizures were less explorative than controls ( $p < .001$ ).

### 3.2.5. Elevated Plus Maze

#### *Animals that survived single seizure on P12*

There was no significant difference between PTZ-treated and saline-treated animals for anxiety score. Two way repeated measures ANOVA for time spent in open vs. closed arms showed no interaction of treatment $\times$ arm [ $F(1, 13) = 0.55, p = .471, \eta^2 = .041$ ] and no significant effect of treatment [ $F(1, 13) = 0.15, p = .708, \eta^2 = .011$ ]. There was a significant main effect of arm [ $F(1,13) = 284.51, p < .001, \eta^2 = .956$ ], indicating that both PTZ-treated ( $N=10$ ) and saline-treated ( $N=7$ ) animals prefer spending time in closed arms (Fig. 10). Two way repeated measures ANOVA for entries into open vs. entries into closed arms showed no interaction of treatment $\times$ arm [ $F(1, 14) = 2.17, p = .163, \eta^2 = .013$ ] and no significant effect of treatment [ $F(1, 14) = 2.53, p = .134, \eta^2 = .153$ ]. There was a significant main effect of arm [ $F(1,14) = 48.72, p < .001, \eta^2 = .777$ ], indicating that both PTZ-treated ( $N=8$ ) and saline-treated ( $N=8$ ) animals show equal preference for the closed arms.



*Figure 10.* Time in open and closed arms in the elevated plus maze. There were no significant differences between the animals with a single seizure on P12 (PTZ-treated) and controls (saline-treated).

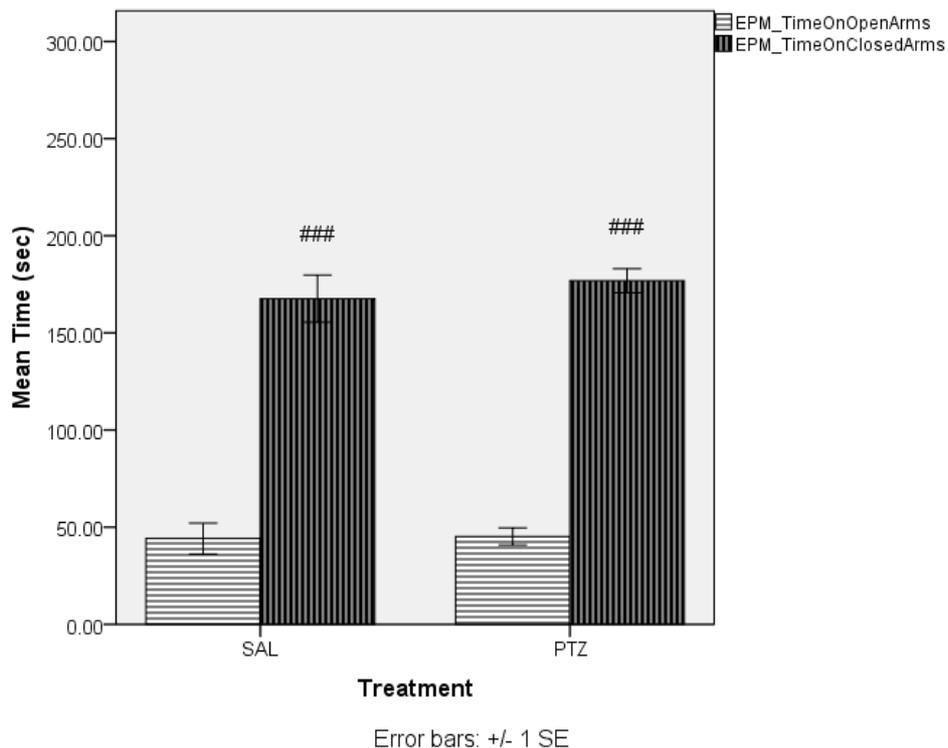
There was no difference in the total number of arm entries [ $t(14) = 1.59, p = .134$ ] or the distance travelled [ $t(17) = .76, p = .457$ ] between PTZ-treated ( $N=8$ ) and saline-treated ( $N=8$ ) animals, indicating that the motor activity was not decreased in PTZ-treated animals in this test.

As far as the percent open-arm time is concerned, no significant difference was found between PTZ-treated ( $N=8$ ) and saline-treated ( $N=7$ ) animals [ $t(13) = 0.85, p = .413$ ], indicating that all animals had the same anxiety score.

#### *Animals that survived single seizure on P22*

No significant difference was found in anxiety score between PTZ-treated and saline-treated animals. As far as the time spent in open vs. closed arms is concerned, two way repeated measures ANOVA showed no interaction of treatment $\times$ arm [ $F(1, 22) = 0.18, p = .678, \eta^2 = .008$ ] and no significant main effect of treatment [ $F(1, 22) = 1.72, p = .203, \eta^2 = .073$ ]. There was a significant main effect of arm [ $F(1,22) = 172.32, p < .001, \eta^2 = .887$ ],

indicating that both PTZ-treated (N=15) and saline-treated (N=9) animals prefer spending time in closed arms (Fig. 11). As far as the entries into open vs. entries into closed arms are concerned, two way repeated measures ANOVA showed no interaction of treatment $\times$ arm [ $F(1, 23) = 0.35, p = .558, \eta^2 = .015$ ] and no significant main effect of treatment [ $F(1, 23) = 4.04, p = .056, \eta^2 = .150$ ]. There was a significant main effect of arm [ $F(1,23) = 29.75, p < .001, \eta^2 = .564$ ], indicating that both PTZ-treated (N=16) and saline-treated (N=9) animals prefer entering into closed arms.



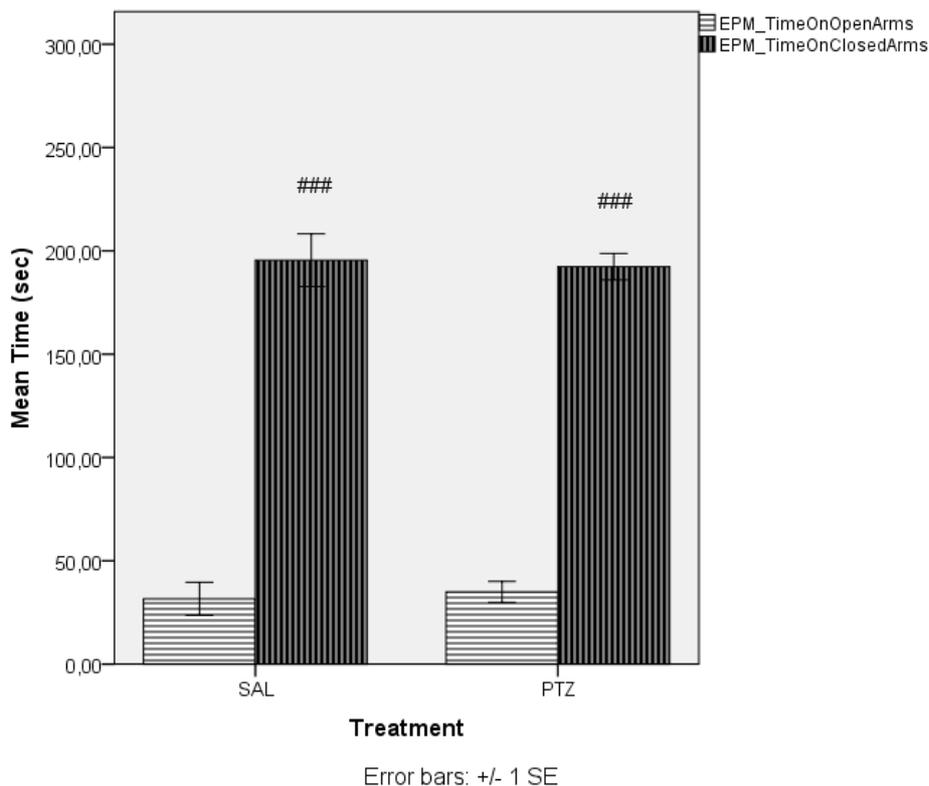
*Figure 11.* Time in open and closed arms in the elevated plus maze. There were no significant differences between the animals with a single seizure on P22 (PTZ-treated) and controls (saline-treated).

There was no difference in the total number of arm entries [ $t(23) = 2.01, p = .056$ ] between PTZ-treated (N=16) and saline-treated (N=9) animals, although there was a significant difference in the distance travelled [ $t(19.9) = 3.91, p = .001$ ] with PTZ-treated animals (N=17) being less active than saline-treated (N=7) animals.

As far as the percent open arm time is concerned, no significant difference was found between PTZ-treated (N=17) and saline-treated (N=9) animals [ $t(24) = 1.75, p = .092$ ].

*Animals that survived multiple seizures on P9-P15*

No significant difference was found in anxiety score between PTZ-treated (N=11) and saline-treated (N=5) animals. As far as the time spent in open vs. closed arms is concerned, two way repeated measures ANOVA showed no interaction of treatment $\times$ arm [ $F(1, 14) = 0.09, p = .764, \eta^2 = .007$ ] and no significant main effect of treatment [ $F(1, 14) = 0.001, p = .978, \eta^2 = .000$ ]. There was a significant main effect of arm [ $F(1,14) = 224.73, p < .001, \eta^2 = .941$ ], indicating that both PTZ-treated and saline-treated animals prefer spending time in closed arms (Fig. 12). As far as the entries into open vs. entries into closed arms are concerned, two way repeated measures ANOVA showed no interaction of treatment $\times$ arm [ $F(1, 14) = 0.01, p = .914, \eta^2 = .001$ ] and no significant main effect of treatment [ $F(1, 14) = 0.02, p = .904, \eta^2 = .001$ ]. There was a significant main effect of arm [ $F(1,14) = 63.66, p < .001, \eta^2 = .820$ ], indicating that both PTZ-treated and saline-treated animals prefer entering into closed arms.



*Figure 12.* Time in open and closed arms in the elevated plus maze. There were no significant differences between the animals with multiple seizures on P9-15 (PTZ-treated) and controls (saline-treated). Both groups had a significant preference for spending time in closed arms.

There was no difference in the total number of arm entries [ $t(10) = -0.29, p = .773$ ] or the distance travelled [ $t(14) = 1.62, p = .128$ ] between PTZ-treated ( $N=7$ ) and saline-treated ( $N=5$ ) animals, indicating that the motor activity was not increased in PTZ-treated animals in this test.

No significant difference was found in percent open arm time between PTZ-treated ( $N=8$ ) and saline-treated ( $N=7$ ) animals [ $t(13) = 0.14, p = .891$ ].

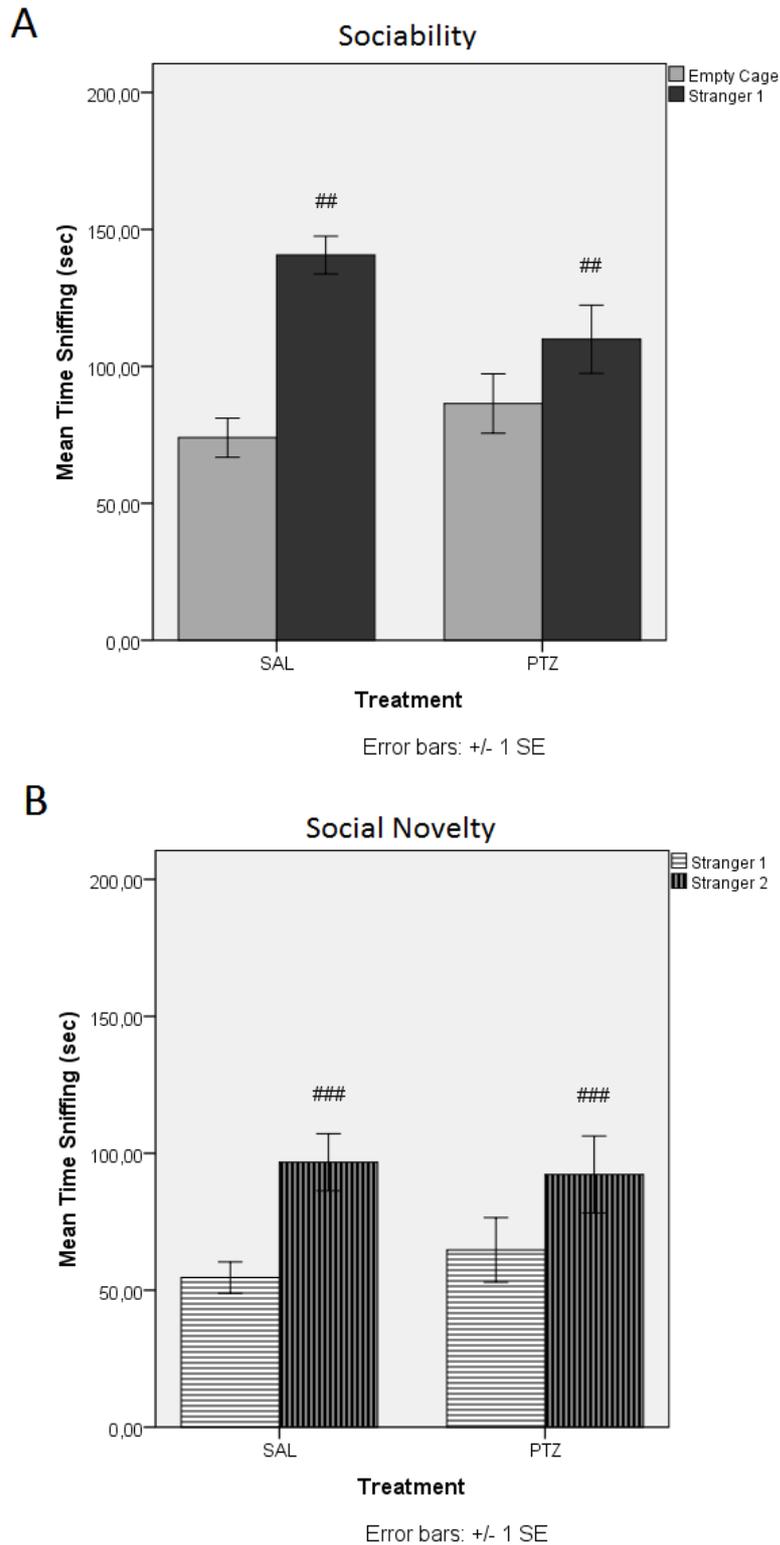
### 3.2.6. *Social behaviour (Sociability and Preference for social novelty tests)*

#### *Animals that survived single seizure on P12*

No significant differences were found between PTZ-treated and saline-treated animals in either the sociability test or preference for social novelty test.

(1) *Habituation*. Two way repeated measures ANOVA for time spent in each empty chamber showed no interaction of chamber $\times$ treatment [ $F(1,15) = 0.16, p = .692, \eta^2 = .011$ ], no significant main effect of treatment [ $F(1, 15) = 0.92, p = .35, \eta^2 = .06$ ] and no significant main effect of chamber [ $F(1, 15) = 0.80, p = .386, \eta^2 = .051$ ].

(2) *Sociability*. No significant difference was found in sociability test between PTZ-treated ( $N=9$ ) and saline-treated ( $N=8$ ) animals. Two way repeated measures ANOVA for time spent in each chamber showed no interaction of chamber $\times$ treatment [ $F(1, 15) = 0.42, p = .526, \eta^2 = .027$ ] and no significant main effect of treatment [ $F(1, 15) = 2.35, p = .146, \eta^2 = .136$ ]. There was no significant main effect of chamber [ $F(1, 15) = 2.71, p = .121, \eta^2 = .153$ ], indicating there was no preference for sociability by neither PTZ-treated nor saline-treated animals. In the same way, two way repeated measures ANOVA for time sniffing each cage showed preference for sociability by all animals. There was no interaction of cage $\times$ treatment [ $F(1, 15) = 3.51, p = .081, \eta^2 = .189$ ] and no significant main effect of treatment [ $F(1, 15) = 0.84, p = .373, \eta^2 = .053$ ], but there was a significant main effect of cage [ $F(1,15) = 15.31, p = .001, \eta^2 = .505$ ], indicating that both PTZ-treated ( $N=10$ ) and saline-treated ( $N=7$ ) animals prefer spending time sniffing the cage containing the animal (stranger1), rather than sniffing the empty cage (Fig. 13A). As far as the number of entries into each chamber is concerned, two way repeated measures ANOVA revealed no interaction of chamber $\times$ treatment [ $F(1, 16) = 0.001, p = .972, \eta^2 = .000$ ] and no significant main effect of chamber [ $F(1, 16) = 0.62, p = .443, \eta^2 = .037$ ], demonstrating no preference for sociability by either PTZ-treated or saline-treated animals. No significant main effect of treatment was found [ $F(1,16) = 3.15, p = .095, \eta^2 = .165$ ], indicating that PTZ-treated animals ( $N=10$ ) were as active as saline-treated ( $N=8$ ) during sociability test.



*Figure 13.* Duration of time sniffing each cage during the test for (A) sociability and (B) preference for social novelty. There were no significant differences between the animals with a single seizure on P12 (PTZ-treated) and controls (saline-treated). Both groups had a significant preference for proximity to Stranger 1 in the test for sociability and a significant preference for proximity to Stranger 2 in the test for social novelty.

(3) *Preference for social novelty.* There were no significant differences in preference for social novelty between PTZ-treated and saline-treated animals. Two way repeated measures ANOVA for time spent in each chamber showed no interaction of chamber×treatment [ $F(1, 15) = 0.38, p = .544, \eta^2 = .025$ ] and no significant main effect of treatment [ $F(1, 15) = 1.62, p = .222, \eta^2 = .098$ ]. A significant main effect of chamber was found [ $F(1,15) = 13.475, p < .01, \eta^2 = .47$ ], indicating that both PTZ-treated (N=9) and saline-treated (N=8) animals preferred spending time in the chamber with the newly introduced animal (stranger 2) in comparison with the chamber with the familiar animal (stranger 1). In the same line, as far as time sniffing each cage is concerned, two way repeated measures ANOVA showed no interaction of cage×treatment [ $F(1, 15) = 1.45, p = .247, \eta^2 = .088$ ] and no significant main effect of treatment [ $F(1, 15) = 0.04, p = .851, \eta^2 = .002$ ], but there was a significant main effect of cage [ $F(1, 15) = 33.19, p < .001, \eta^2 = .689$ ], demonstrating there was a preference by both PTZ-treated (N=9) and saline-treated (N=8) animals for sniffing the new animal (stranger 1) rather than the familiar one (stranger 2) (Fig. 13B). Significant preference for social novelty was not observed for the number of entries into each chamber. Two way repeated measures ANOVA revealed no interaction of chamber×treatment [ $F(1, 15) = 0.003, p = .955, \eta^2 = .000$ ] and no significant main effect of chamber [ $F(1, 15) = 0.45, p = .511, \eta^2 = .029$ ]. There was a significant main effect of treatment [ $F(1, 15) = 8.67, p = .010, \eta^2 = .366$ ], indicating that PTZ-treated animals (N=9) were less active than saline-treated (N=8) during preference for social novelty test.

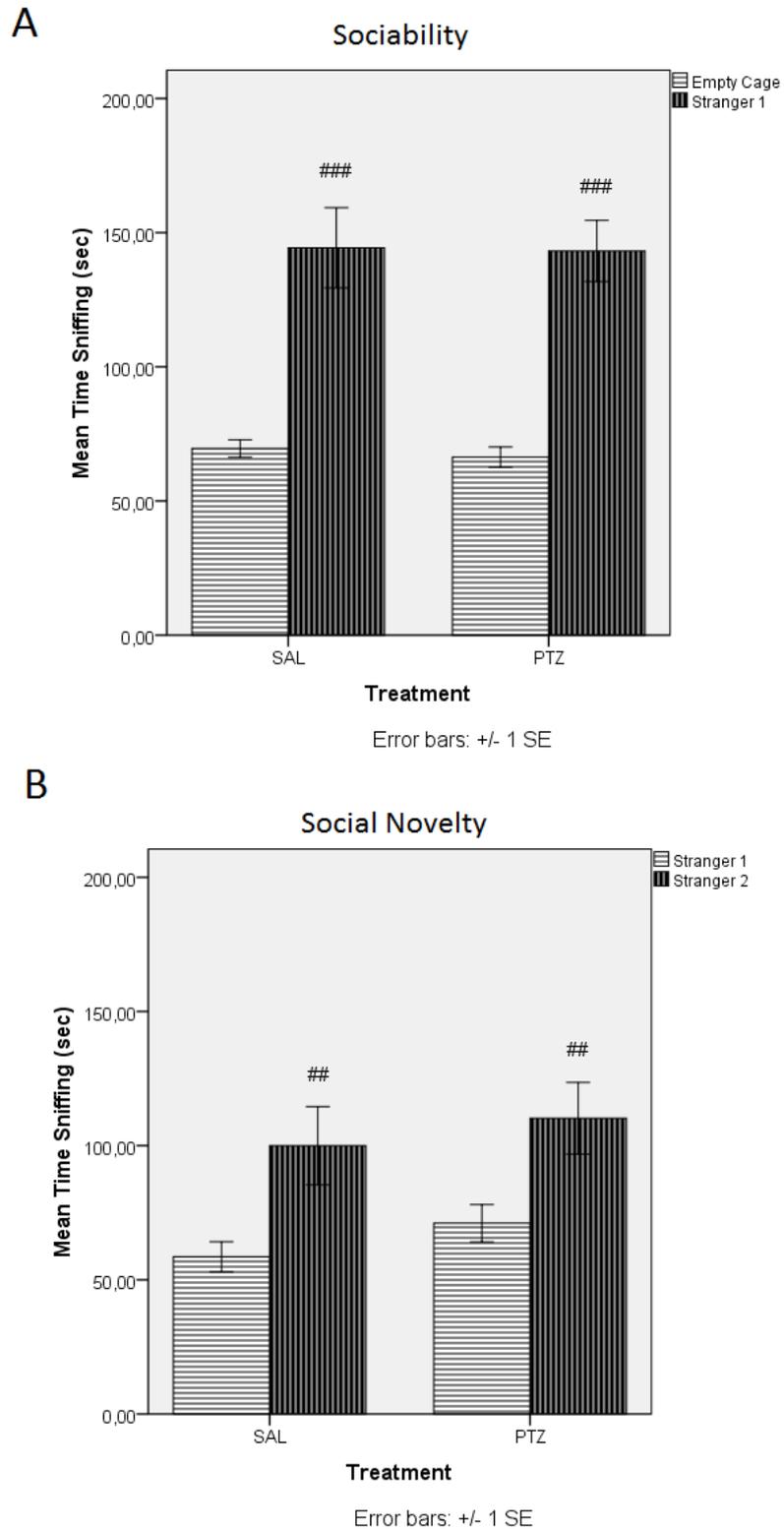
#### *Animals that survived single seizure on P22*

No significant differences were found between PTZ-treated and saline-treated animals in either the sociability test or preference for social novelty test.

(1) *Habituation.* Two way repeated measures ANOVA for time spent in each empty chamber showed no interaction of chamber×treatment [ $F(1, 21) = 0.03, p = .859, \eta^2 = .002$ ] and no significant main effect of treatment [ $F(1, 21) = 0.72, p = .406, \eta^2 = .033$ ]. Unexpectedly, a significant main effect of chamber [ $F(1,21) = 21.17, p < .001, \eta^2 = .502$ ] was found, indicating that both experimental and control animals spent more time in the left empty chamber. This result is believed not to influence the results of the sociability and social

novelty test, since the positions of the empty cage, the stranger1 and the stranger 2 were systematically alternated.

(2) *Sociability*. No significant difference was found in sociability test between PTZ-treated (N=9) and saline-treated (N=6) animals. Two way repeated measures ANOVA for time spent in each chamber showed no interaction of chamber×treatment [ $F(1, 21) = 0.02, p = .905, \eta^2 = .001$ ] and no significant main effect of treatment [ $F(1, 21) = 4.15, p = .054, \eta^2 = .165$ ]. There was a significant main effect of chamber [ $F(1,21) = 17.85, p < .001, \eta^2 = .459$ ], indicating preference for sociability by both PTZ-treated and saline-treated animals. In the same way, two way repeated measures ANOVA for time sniffing each cage showed preference for sociability by all animals. There was no interaction of cage×treatment [ $F(1, 17) = 0.01, p = .924, \eta^2 = .001$ ] and no significant main effect of treatment [ $F(1, 17) = 0.05, p = .831, \eta^2 = .003$ ], but there was a significant main effect of cage [ $F(1,17) = 35.49, p < .001, \eta^2 = .676$ ], indicating that both PTZ-treated (N=14) and saline-treated (N=5) animals prefer spending time sniffing the cage containing the animal (stranger1), rather than sniffing the empty cage (Fig. 14A). As far as the number of entries into each chamber is concerned, two way repeated measures ANOVA revealed no interaction of chamber×treatment [ $F(1, 21) = 0.003, p = .954, \eta^2 = .000$ ] and no significant main effect of chamber [ $F(1, 21) = 3.21, p = .087, \eta^2 = .133$ ], demonstrating no preference for sociability. No significant main effect of treatment was found [ $F(1,21) = 0.84, p = .369, \eta^2 = .039$ ], indicating that PTZ-treated animals (N=15) were as active as saline-treated (N=8).



*Figure 14.* Duration of time sniffing each cage during the test for (A) sociability and (B) preference for social novelty. There were no significant differences between the animals with a single seizure on P22 (PTZ-treated) and controls (saline-treated). Both groups had a significant preference for proximity to Stranger 1 in the test for sociability and a significant preference for proximity to Stranger 2 in the test for social novelty.

(3) *Preference for social novelty.* There were no significant differences in preference for social novelty between PTZ-treated and saline-treated animals. Two way repeated measures ANOVA for time spent in each chamber showed no interaction of chamber×treatment [ $F(1, 22) = 0.28, p = .602, \eta^2 = .013$ ] and no significant main effect of treatment [ $F(1, 22) = 2.22, p = .150, \eta^2 = .092$ ]. A significant main effect of chamber was found [ $F(1,22) = 5.31, p < .05, \eta^2 = .195$ ], indicating that both PTZ-treated (N=17) and saline-treated (N=7) animals preferred spending time in the chamber with the newly introduced animal (stranger 2) in comparison with the chamber with the familiar animal (stranger 1). In the same line, as far as time sniffing each cage is concerned, two way repeated measures ANOVA showed no interaction of cage×treatment [ $F(1, 20) = 0.01, p = .932, \eta^2 = .000$ ] and no significant main effect of treatment [ $F(1, 20) = 0.71, p = .408, \eta^2 = .034$ ], but there was a significant main effect of cage [ $F(1,20) = 9.39, p < .01, \eta^2 = .319$ ], demonstrating there was a preference by both PTZ-treated (N=16) and saline-treated (N=6) animals for sniffing the new animal (stranger 1) rather than the familiar one (stranger 2) (Fig. 14B). Significant preference for social novelty was not observed for the number of entries into each chamber. Two way repeated measures ANOVA revealed no interaction of chamber×treatment [ $F(1, 22) = 0.99, p = .331, \eta^2 = .043$ ], no significant main effect of chamber [ $F(1, 22) = 0.54, p = .472, \eta^2 = .024$ ] and no significant main effect of treatment [ $F(1,22) = 0.02, p = .892, \eta^2 = .001$ ].

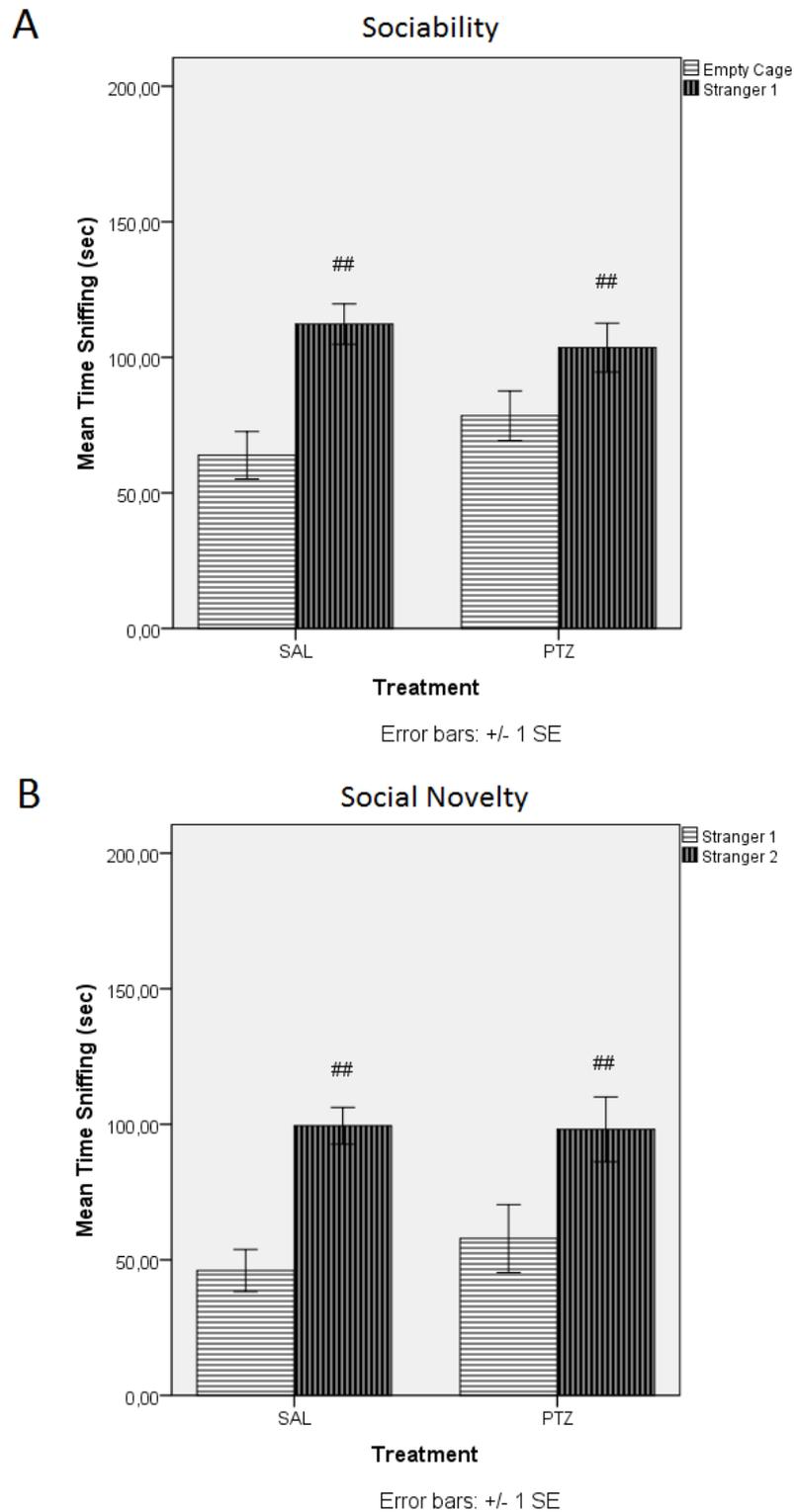
#### *Animals that survived multiple seizures on P9-P15*

No significant differences were found between PTZ-treated and saline-treated animals in either the sociability test or preference for social novelty test.

(1) *Habituation.* Two way ANOVA for time spent in each empty chamber showed no interaction of chamber×treatment [ $F(1, 14) = 0.02, p = .896, \eta^2 = .001$ ] and no significant main effect of treatment [ $F(1, 14) = 0.22, p = .643, \eta^2 = .016$ ]. Unexpectedly, a significant main effect of chamber [ $F(1,14) = 8.01, p < .05, \eta^2 = .364$ ] was found, indicating that both experimental and control animals spent more time in the left empty chamber. This result is believed not to influence the results of the sociability and social novelty test, since the positions of the empty cage, the stranger1 and the stranger2 were systematically alternated.

(2) *Sociability.* No significant difference was found in sociability test between PTZ-treated (N=9) and saline-treated (N=6) animals. Two way repeated measures ANOVA for time spent in each chamber showed no interaction of chamber×treatment [ $F(1, 13) = 4.49, p =$

.054,  $\eta^2 = .257$ ], no significant main effect of treatment [ $F(1, 13) = 0.99$ ,  $p = .337$ ,  $\eta^2 = .071$ ] and no significant main effect of chamber [ $F(1,13) = 0.003$ ,  $p = .957$ ,  $\eta^2 = .000$ ], indicating no preference for sociability. On the other hand, two way repeated measures ANOVA for time sniffing each cage showed preference for sociability by all animals. There was no interaction of cage $\times$ treatment [ $F(1, 15) = 0.89$ ,  $p = .359$ ,  $\eta^2 = .056$ ] and no significant main effect of treatment [ $F(1, 15) = 0.23$ ,  $p = .640$ ,  $\eta^2 = .015$ ], but there was a significant main effect of cage [ $F(1,15) = 8.96$ ,  $p < .01$ ,  $\eta^2 = .374$ ], indicating that both PTZ-treated (N=11) and saline-treated (N=6) animals prefer spending time sniffing the cage containing the animal (stranger1), rather than sniffing the empty cage (Fig. 15A). As far as the number of entries into each chamber is concerned, two way repeated measures ANOVA revealed no interaction of chamber $\times$ treatment [ $F(1, 15) = 0.57$ ,  $p = .461$ ,  $\eta^2 = .037$ ] and no significant main effect of chamber [ $F(1, 15) = 0.01$ ,  $p = .907$ ,  $\eta^2 = .001$ ], demonstrating no preference for sociability. There was a significant main effect of treatment [ $F(1,15) = 11.79$ ,  $p < .01$ ,  $\eta^2 = .440$ ], indicating that PTZ-treated animals (N=11) were less active than saline-treated (N=6).



*Figure 15.* Duration of time sniffing each cage during the test for (A) sociability and (B) preference for social novelty. There were no significant differences between the animals with multiple seizures on P9-15 (PTZ-treated) and controls (saline-treated). Both groups had a significant preference for proximity to Stranger 1 in the test for sociability and a significant preference for proximity to Stranger 2 in the test for social novelty.

(3) *Preference for social novelty*. There were no significant differences in preference for social novelty between PTZ-treated and saline-treated animals. Two way repeated measures ANOVA for time spent in each chamber showed no interaction of chamber×treatment [ $F(1, 16) = 0.10, p = .757, \eta^2 = .006$ ] and no significant main effect of treatment [ $F(1, 16) = 0.11, p = .748, \eta^2 = .007$ ]. A significant main effect of chamber was found [ $F(1,16) = 17.91, p < .01, \eta^2 = .528$ ], indicating that both PTZ-treated (N=11) and saline-treated (N=7) animals preferred spending time in the chamber with the newly introduced animal (stranger 2) in comparison with the chamber with the familiar animal (stranger 1). In the same line, as far as time sniffing each cage is concerned, two way repeated measures ANOVA showed no interaction of cage×treatment [ $F(1, 15) = 0.32, p = .583, \eta^2 = .021$ ] and no significant main effect of treatment [ $F(1, 15) = 0.23, p = .637, \eta^2 = .015$ ], but there was a significant main effect of cage [ $F(1,15) = 16.03, p < .01, \eta^2 = .517$ ], demonstrating there was a preference by both PTZ-treated (N=10) and saline-treated (N=7) animals for sniffing the new animal (stranger 1) rather than the familiar one (stranger 2) (Fig. 15B). Significant preference for social novelty was not observed for the number of entries into each chamber. Two way repeated measures ANOVA revealed no interaction of chamber×treatment [ $F(1, 17) = 0.03, p = .865, \eta^2 = .002$ ] and no significant main effect of chamber [ $F(1, 17) = 1.27, p = .276, \eta^2 = .069$ ]. Though, there was a significant main effect of treatment [ $F(1,17) = 8.45, p < .05, \eta^2 = .332$ ], indicating that PTZ-treated animals (N=12) were less active than saline-treated (N=7).

### 3.2.7. Rotarod

#### *Animals that survived single seizures on P12*

No significant difference was found between PTZ-treated (N=10) and saline-treated (N=9) animals in latency to fall off the rod during the Rotarod test. Student's t-test for the average latency value calculated from all three trials (Saline-treated:  $106.9 \pm 17.9$  vs PTZ-treated:  $133.5 \pm 66.7$ ) showed no difference [ $t(17) = -0.95, p = .355$ ].

#### *Animals that survived single seizures on P22*

No significant difference was found between PTZ-treated (N=17) and saline-treated (N=9) animals in latency to fall off the rod during the Rotarod test. Student's t-test for the average latency value calculated from all three trials (Saline-treated:  $138.2 \pm 12.6$  vs PTZ-treated:  $187.5 \pm 16.9$ ) showed no difference [ $t(24) = -1.96, p = .061$ ].

*Animals that survived multiple seizures on P9-P15*

No significant difference was found between PTZ-treated (N=12) and saline-treated (N=7) animals in latency to fall off the rod during the Rotarod test. Student's t-test for the average latency value calculated from all three trials (Saline-treated:  $111.6 \pm 21.9$  vs PTZ-treated:  $165.8 \pm 22.65$ ), showed no difference [  $t(17) = -1.6, p = .13$ ].

**3.2.8. Novel object recognition task***Animals that survived single seizures on P12**Day 1.*

Examining the distance traveled during the two 10 min trials of the habituation day, two way repeated measures ANOVA showed no interaction of trial×treatment [ $F(1, 17) = 0.69, p = .418, \eta^2 = .039$ ]. There was a significant main effect of trial [ $F(1, 17) = 25.69, p < .001, \eta^2 = .602$ ], indicating a reduced locomotor activity by both PTZ-treated (N=10) and saline-treated (N=9) animals from first trial to second trial. No significant main effect of treatment was found [ $F(1, 17) = 0.51, p = .485, \eta^2 = .029$ ], indicating that during both trials the PTZ-treated animals showed the same levels of ambulatory distance as the saline-treated animals.

No differences between PTZ-treated (N=10) and saline-treated (N=9) animals were found in the time spent in the center region of the arena. Two way repeated measures ANOVA showed no interaction of trial×treatment [ $F(1, 17) = 1.71, p = .208, \eta^2 = .091$ ], no significant main effect of trial [ $F(1, 17) = 0.59, p = .453, \eta^2 = .034$ ], and no significant main effect of treatment [ $F(1, 17) = 4.07, p = .060, \eta^2 = .193$ ].

*Day 2.*

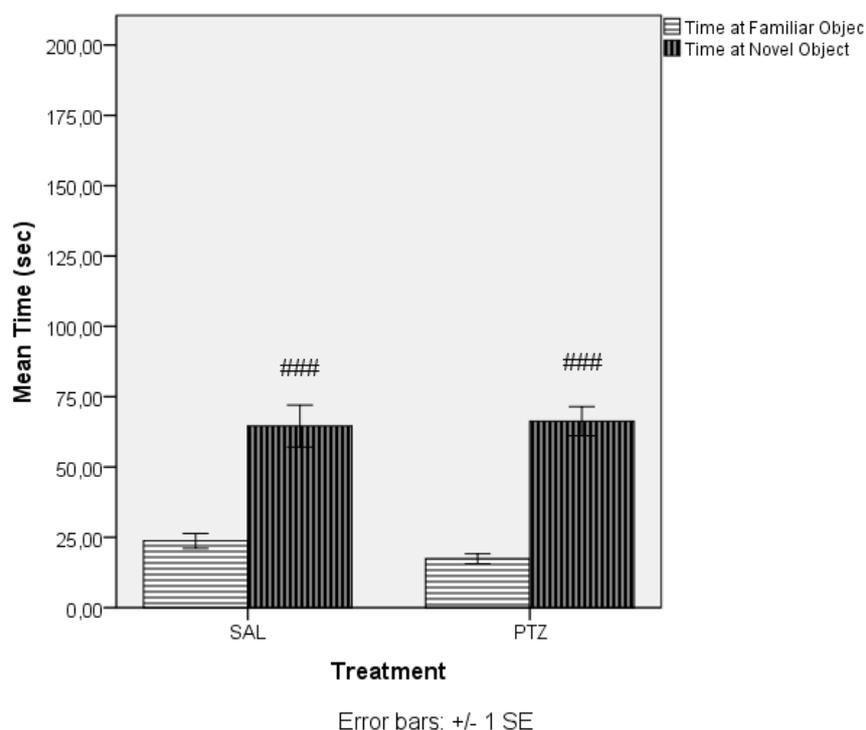
Two way ANOVA for time spent sniffing each of the two identical objects during their first presentation, showed no interaction of object×treatment [ $F(1, 17) = 0.12, p = .737, \eta^2 = .007$ ], no significant main effect of object [ $F(1, 17) = 0.03, p = .858, \eta^2 = .002$ ] and no significant main effect of treatment [ $F(1, 17) = 0.01, p = .937, \eta^2 = .000$ ].

*Day 3.*

Two way ANOVA for time spent sniffing each of the familiar and novel objects during the testing trial showed no interaction of object×treatment [ $F(1, 16) = 1.22, p = .285, \eta^2 = .071$ ] and no significant main effect of treatment [ $F(1, 16) = 0.16, p = .695, \eta^2 = .010$ ].

There was a significant main effect of object [ $F(1, 16) = 152.06, p < .001, \eta^2 = .905$ ], indicating that both PTZ-treated ( $N=9$ ) and saline-treated ( $N=9$ ) animals spent more time sniffing the novel object rather than the familiar one (Fig. 16).

As far as the discrimination index is concerned, there was a difference between PTZ-treated and saline-treated animals [ $t(16) = -2.73, p < .05$ ], demonstrating that PTZ-treated animals ( $N=9$ ) had a higher discrimination index compared with saline-treated animals ( $N=9$ ).



*Figure 16.* Duration of time sniffing each object during the test for novel object recognition. There were no significant differences between the animals with a single seizure on P12 (PTZ-treated) and controls (saline-treated). Both groups had a significant preference for sniffing novel object.

#### *Animals that survived single seizures on P22*

##### *Day 1.*

Examining the distance traveled during the two 10 min trials of the habituation day, two way repeated measures ANOVA showed no interaction of trial×treatment [ $F(1, 23) = 0.29, p = .594, \eta^2 = .013$ ]. There was no significant main effect of treatment [ $F(1, 23) = 0.03, p =$

.869,  $\eta^2 = .001$ ], indicating that during both trials, PTZ-treated (N=9) and saline-treated (N=17) animals had the same levels of ambulatory distance. There was a significant main effect of trial [ $F(1, 23) = 101.96$ ,  $p < .001$ ,  $\eta^2 = .816$ ], indicating a reduced activity by both PTZ-treated and saline-treated animals from first trial to second trial.

No differences between PTZ-treated (N=17) and saline-treated (N=7) animals were found in the time spent in the center region of the arena. Two way repeated measures ANOVA showed no interaction of trial×treatment [ $F(1, 22) = 1.36$ ,  $p = .256$ ,  $\eta^2 = .058$ ], no significant main effect of trial [ $F(1, 22) = 3.10$ ,  $p = .092$ ,  $\eta^2 = .124$ ], and no significant main effect of treatment [ $F(1, 22) = 0.01$ ,  $p = .919$ ,  $\eta^2 = .000$ ].

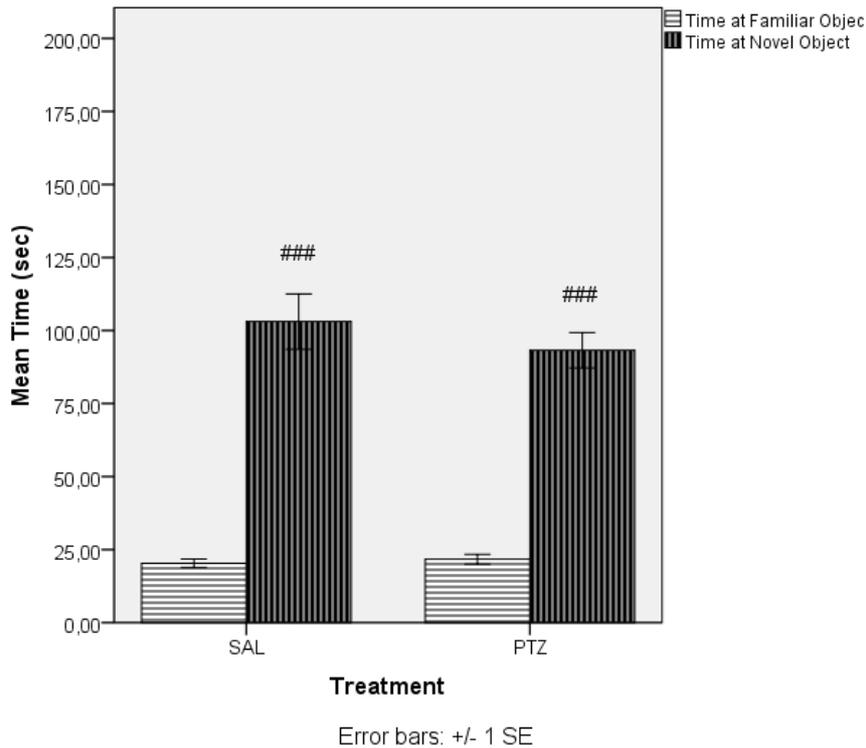
#### Day 2.

Two way ANOVA for time spent sniffing each of the two identical objects during their first presentation, showed no interaction of object×treatment [ $F(1, 24) = 0.02$ ,  $p = .886$ ,  $\eta^2 = .001$ ], no significant main effect of object [ $F(1, 24) = 0.99$ ,  $p = .327$ ,  $\eta^2 = .040$ ] and no significant main effect of treatment [ $F(1, 24) = 0.24$ ,  $p = .630$ ,  $\eta^2 = .010$ ].

#### Day 3.

Two way ANOVA for time spent sniffing each of the familiar and novel objects during the testing trial revealed no interaction of object×treatment [ $F(1, 23) = 0.99$ ,  $p = .331$ ,  $\eta^2 = .041$ ] and no significant main effect of treatment [ $F(1, 23) = 0.62$ ,  $p = .439$ ,  $\eta^2 = .026$ ]. There was a significant main effect of object [ $F(1, 23) = 186.82$ ,  $p < .001$ ,  $\eta^2 = .890$ ], indicating that both PTZ-treated and saline-treated animals spent more time sniffing the novel object rather than the familiar one (Fig. 17).

As far as the discrimination index is concerned, no difference was found between PTZ-treated and saline-treated animals [ $t(23) = 1.95$ ,  $p = .063$ ].



*Figure 17.* Duration of time sniffing each object during the test for novel object recognition. There were no significant differences between the animals with a single seizure on P22 (PTZ-treated) and controls (saline-treated). Both groups had a significant preference for sniffing novel object.

#### *Animals that survived multiple seizures on P9-P15*

##### *Day 1.*

Examining the distance traveled during the two 10 min trials of the habituation day, two way repeated measures ANOVA showed no interaction of trial×treatment [ $F(1, 16) = 1.72, p = .208, \eta^2 = .097$ ]. There was a significant main effect of treatment [ $F(1, 16) = 11.49, p < .01, \eta^2 = .418$ ], indicating that during both trials the PTZ-treated animals ( $N=12$ ) had significantly lower levels of ambulatory distance than the saline-treated ( $N=6$ ) animals. There was also a significant main effect of trial [ $F(1, 16) = 150.20, p < .001, \eta^2 = .904$ ], indicating a reduced activity by both PTZ-treated and saline-treated animals from first trial to second trial.

No differences between PTZ-treated ( $N=10$ ) and saline-treated ( $N=9$ ) animals were found in the time spent in the center region of the arena. Two way repeated measures ANOVA showed no interaction of trial×treatment [ $F(1, 16) = 1.26, p = .279, \eta^2 = .073$ ] and no significant main effect of treatment [ $F(1, 16) = 0.06, p = .802, \eta^2 = .004$ ]. There was a

significant main effect of trial [ $F(1, 16) = 17.10, p = .001, \eta^2 = .517$ ], indicating that the time spent in the center was reduced from first trial to second trial for both PTZ-treated and saline-treated animals.

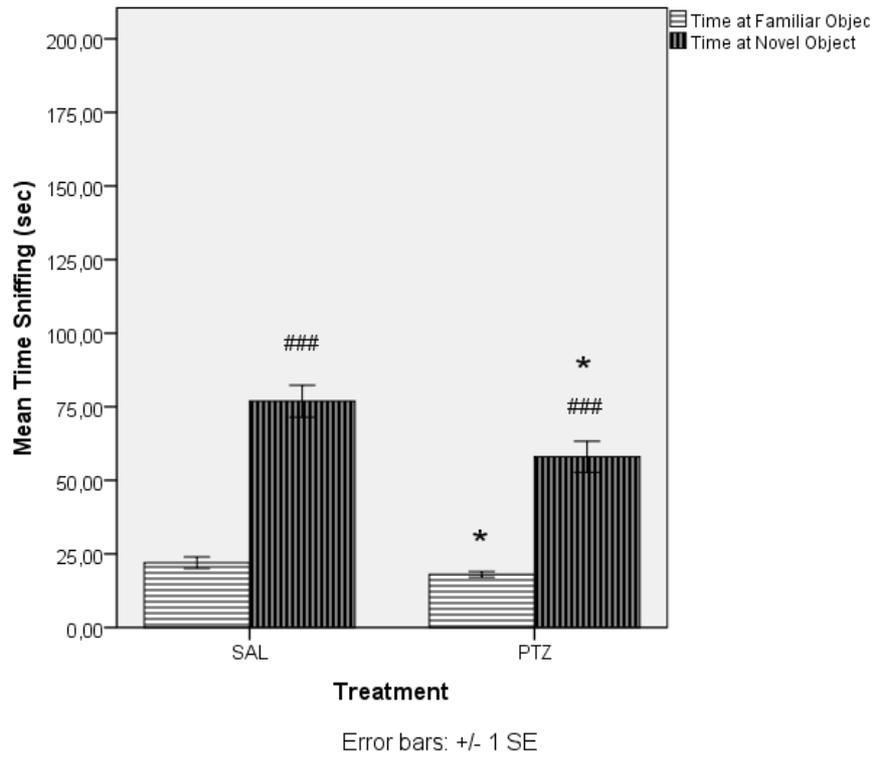
#### *Day 2.*

Two way ANOVA for time spent sniffing each of the two identical objects during their first presentation, showed no interaction of object $\times$ treatment [ $F(1, 17) = 0.27, p = .610, \eta^2 = .016$ ], no significant main effect of object [ $F(1, 17) = 3.34, p = .085, \eta^2 = .164$ ] and no significant main effect of treatment [ $F(1, 17) = 0.01, p = .922, \eta^2 = .001$ ].

#### *Day 3.*

Two way ANOVA for time spent sniffing each of the familiar and novel objects during the testing trial showed no interaction of object $\times$ treatment [ $F(1, 15) = 3.63, p = .076, \eta^2 = .195$ ]. There was a significant main effect of object [ $F(1, 15) = 146.66, p < .001, \eta^2 = .907$ ], indicating that both PTZ-treated (N=10) and saline-treated (N=7) animals spent more time sniffing the novel object rather than the familiar one. There was also a significant main effect of treatment [ $F(1, 15) = 7.75, p < .05, \eta^2 = .341$ ], demonstrating that PTZ-treated animals (N=10) spent less time sniffing both objects, in comparison with saline-treated animals (N=7) (Fig. 18).

As far as the discrimination index is concerned, no difference was found between PTZ-treated (N=12) and saline-treated (N=7) animals [ $t(17) = 1.25, p = .228$ ].



*Figure 18.* Duration of time sniffing each object during the test for novel object recognition. There were no significant differences between the animals with multiple seizures on P9-15 (PTZ-treated) and controls (saline-treated). PTZ-treated animals spent less time sniffing both objects. Both groups had a significant preference for sniffing novel object.

## 4. Discussion

Seizures affect at least 2% of the population and although they can occur at any age, the highest incidence of them occurs during the first years of life (Holmes and Ben-Ari, 1998). Early-life seizures are often associated with neurological and behavioural impairments in adult life, while antiepileptic drugs can also have cognitive side-effects, making the dilemma whether to treat early non-persistent seizures a serious clinical issue. As a result, the cognitive long-term effects of early-life seizures need to be investigated in depth and this is something that can be more reliably carried out through animal studies.

The purpose of this thesis was to examine the behavioural/cognitive long-term effects of either single or multiple seizures occurring at two different developmental stages. Our hypothesis was that mice with a history of early life seizures may exhibit deficits in adult life. In particular we wanted to examine if distinct developmental periods have differential sensitivity to the effects of early seizures (single seizure at P9-15 vs. P19-25), and whether mice exposed to multiple seizures during the same developmental window would show increased deficits

Few studies have investigated the long-term effects of seizures occurring at different developmental stages while conducting a variety of behavioural experiments to test both cognitive and motor function in adulthood. Specifically, Karnam et al. (2009a) induced seizures in animals at P0-P10 and P15-P25 and tested them later in life – using Morris water maze, radial arm water maze, open-field and active avoidance. Koh et al. (1999) induced seizures in animals at P15 and P45 and tested them at P50 only in modified Morris water maze. Kornelsen et al. (1996) induced seizures in animals at P1, P10 and P21 and only used Digiscan Activity monitors and water maze for their assessment a few months later. Liu et al. (1994) induced seizures at P20 and P45 animals and tested them in adulthood only in open-field and water maze. Nishimura et al. (2011) induced seizures in animals at P7-P11 and P30-P34 and tested them at P50-P60 only in water maze. Sayin et al. (2004) induced seizures in animals at P1, P7, P14 and P24 and tested them in adulthood in Morris water maze, radial arm water maze, elevated plus maze and open-field. The current study investigates two different, very important for the development ages at which the seizures are induced and covers a wide range of behavioural tests, including tests that are used for first time in order to evaluate long-term effects of early-life seizures. Moreover, the most suitable protocols for seizure induction and seizure severity assessment are used in this study, as some previous

studies could receive criticism for using seizure induction methods that have side effects or for not presenting a complete seizure severity assessment.

The data of the current study mainly showed that all groups that experienced early-life seizures were more hypoactive and less explorative, without presenting further cognitive deficits, when compared to control animals. Locomotor activity and exploratory behaviour in open-field were evaluated and all experimental groups expressed hypoactivity as a decrease in horizontal activity (distance travelled) and reduced exploratory behaviour as a decrease in vertical activity (rearings). In addition, reduced horizontal activity was also found in the present study for the experimental animals that experienced multiple seizures at P9-15 compared to controls, during the first day (day of habituation) of the novel object recognition test. The above findings are consistent with those of Holmes et al. (1998), Koh et al. (2005) and Kubova et al. (2000). Specifically, in the study of Kubova et al. (2000), they found reduced locomotor and exploratory activity for an experimental group that received seizures at P12, while an experimental group that received seizures at P25 showed increased locomotor and exploratory activity, inconsistent with the present findings. These findings are also inconsistent with those of Santos et al. (2000b) and Kubova et al. (2004), in which experimental groups showed higher locomotor and exploratory activity compared with control groups. Possible reasons for the discrepancy among the results of the above studies including the current study are the different drugs used for the seizures induction, the different ages at which the seizures are induced and the different ages at which the behavioural tests are conducted. In particular, Holmes et al. (1998) used flurothyl, Koh et al. (2005) used kainic acid, the current study used pentylenetetrazole, while, on the other hand, Santos et al. (2000b) and Kubova et al. (2004) used pilocarpine. Holmes et al. (1998) induced seizures at P0-P4, Koh et al. (2005) at postnatal days between P20 and P25, while Santos et al. (2000b) induced seizures at P7-P9 and Kubova et al. (2004) at P12 and P25. In the cases of Holmes et al. (1998) and Koh et al. (2005) the behavioural tests were done between P27 and P32, while in the cases of Santos et al. (2000b) and Kubova et al. (2004) the tests were done between P60 and P90. Obviously, no conclusion can be made about how the above variables (seizure induction protocol, age of seizures, age of behavioural tests) can have as a long-term effect either hypoactivity or hyperactivity.

The lower levels of locomotor activity found in the experimental animals in this thesis cannot be ascribed to purely motor impairment, as no deficit in motor performance was found in the rotarod test. The results in the rotarod test are consistent with previous studies (Huang et al., 2002; Kubova et al., 2000; Santos et al., 2000b). This suggests that the differences in

the locomotor activity between animals with a history of early-life seizures and animals with no experience of seizures could be due to changes in brain regions responsible for the locomotor activity, as the motor cortex or the cerebellum.

In contrast with Karnam et al. (2009a), experimental animals showed similar habituation to control animals in the novel open-field test, as expressed by the gradual reduction of distance traveled over time. Habituation is the simplest form of implicit learning and involves changes in the sensory and motor systems involved in the learning. In habituation, an animal first responds to a novel stimulus (novel environment) by attending to it with a series of orienting responses. If the stimulus is neither beneficial nor harmful, the animal learns to ignore it. Thus, in the current study, experimental animals did not seem to have any deficit in implicit learning. Reasons for the disagreement between this result and the result of Karnam et al. (2009a) who found that neither of their two experimental groups had a reduction in activity level during the open-field test, may be the different protocol for seizures induced and/or the different protocol for the open-field test. Specifically, Karnam et al. (2009a) induced 50 seizures in total (5 short in duration seizures per day) at P0-P10 or P15-P25 and the drug they used was flurothyl. Also, they tested the animals in the open-field at P60-P61 for two minutes per day. Obviously, there are many differences in the methods followed which make the comparison of the studies and their results difficult.

Another interesting finding of this study is the deficit observed in every-day spontaneous activities, as assessed in the marble-burying test. Specifically, experimental animals that experienced single or multiple seizures during the earlier developmental period (P9-15) showed reduced burying of marbles when compared to their controls. However, this decrease in marble burying may be due to the hypoactivity that these experimental animals presented during the marble burying test. Specifically, a high correlation was found between the percent of marbles buried and the distance travelled during this test for the groups of single and multiple seizures at P9-15. To my knowledge, this is the first study to conduct the marble-burying test in order to evaluate the long-term effects of early-life seizures.

Anxiety-like behaviour in animals that experienced early-life seizures did not differ when compared to controls. In contrast with studies that have shown either increased (Sayin et al., 2004; Kubova et al., 2004) or decreased (Santos et al., 2000b) anxiety-like behaviour in adult animals that have experienced early-life seizures, this study found no differences between experimental and control animals in anxiety score as measured with the elevated plus maze or the time spent in the center of the open-field. Other studies that also found no differences in anxiety score between animals with a history of seizures and control animals

are those of Cognato et al. (2010) and Cornejo et al. (2008). Both of these studies used kainate to induce one seizure at P7. Sayin et al. (2004) also used kainate to induce one seizure at P1 or P7 or P14 or P24, while Kubova et al. (2004) used pilocarpine to induce one seizure at P25. On the other hand, Santos et al. (2000) also used pilocarpine to induce three seizures at P7-P9. All the above studies tested for five minutes the animals in elevated plus maze during adulthood. As a result, based on the current literature, there are not any obvious experimental conditions that can explain the differences in anxiety score

Results for object recognition memory are consistent with Cornejo et al. (2008), as there was no deficit in animals that experienced early-life seizures when compared to controls in the novel object recognition test. Future studies should examine additional aspects of recognition memory, ie. short-term and long-term memory retrieval and storage processes.

No deficit in social behaviour was observed in experimental animals when compared to controls, as was shown in the sociability and preference for social novelty tests. To my knowledge, there is no study that has conducted the social behaviour tests to evaluate the long-term effects of early-life seizures.

In conclusion, experimental animals that experienced early-life seizures exhibited hypoactivity, reduced exploration and poorer performance in marble-burying behaviour. Interestingly, they did not show any further cognitive dysfunction regarding social behaviour and recognition memory.

Presently, we can not comment on the differential sensitivity to early seizures at the two developmental ages. Comparisons between experimental groups of the different developmental periods could not be made due to the fact that there were statistically significant differences between the control groups in several tests. This may be explained by differences in generations of animals or by the fact that experiments were conducted during different seasons of the year for the different groups of animals. Thus, the results of the thesis are focused on differences between experimental and control groups within the same developmental window of either single or multiple seizures case.

Future work is necessary to characterize the long-term behavioural impact of early-life seizures. Future studies must elucidate under which conditions (time of seizures, number of seizures, time of test, etc) locomotor activity is affected. A group that will experience multiple seizures at P19-25 must also be evaluated for long-term behavioural effects. The current seizure induction method which uses pentylenetetrazole has to be compared with other induction methods, such as the use of flurothyl or hyperthermia, keeping though all other parameters identical, in order to assess whether there are any differences in behaviour

experimental results which come from the induction protocol itself. Such a finding would make it mandatory to better evaluate which seizure induction protocol is more appropriate to follow. Based on previous studies that have used the water maze test for the evaluation of spatial memory and learning and have shown cognitive deficits following early-life seizures, the current findings must be extended to include this test for the sake of comparison and consistency. Certainly, the effects of antiepileptic drugs on behaviour must also be investigated in depth and compared to the effects of non-treated seizures, so as to conclude if it is better or not to treat these early-life seizures. Additional groups with the experience of early-life seizures that will be exposed to a stressful experience (psychological or physical) during adolescence could also be evaluated for possible seizure-induced vulnerability that is not apparent under basal conditions. Overall, future research is critical to further our understanding of the impact of early-life seizures on later adulthood for better treatment intervention.

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