Evaluation of the effects of liraglutide on the development of epilepsy and behavioural alterations in two animal models of epileptogenesis

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Abstract

Liraglutide (LIR) is a novel long-lasting glucagon-like peptide-1 (GLP-1) analogue that facilitates insulin signalling and shows also neuroprotective properties in different brain disease models. In this study, we explored the potential antiepileptogenic effects of LIR in two different animal models; namely, the mouse intrahippocampal kainic acid (KA) model of temporal lobe epilepsy and the WAG/Rij rat model of absence epileptogenesis. Moreover, we evaluated LIR effects on comorbidities in various behavioural tests. Mice with kainate-induced epilepsy were treated with LIR (300 μg/kg/day s.c.) for 4 weeks after *status epilepticus* and then evaluated for drug effects on seizure development and behavioural alterations, whereas WAG/Rij rats were treated for 17 weeks (starting at 30 days of age, before seizure onset) with LIR (300 μg/kg/day s.c.) in order to investigate whether an early chronic treatment was able to reduce the development of absence seizures and related comorbidities. Our results indicate that LIR was effective in reducing the development of spontaneous seizures in kainate-induced epilepsy; moreover, in this model, it prevented memory impairment and related anxiety-like behaviour in the open field (OF) test while in the forced swimming test (FST), LIR displayed an apparent pro-depressant effect that was instead related to reduced endurance as confirmed by rotarod test. In contrast, LIR was unable to modify the epileptogenic process underlying the development of absence seizures in WAG/Rij rats while being antidepressant in the FST in this strain. Our results indicate that LIR may represent a promising novel treatment to prevent and treat the epileptogenic process and its associated behavioural and cognitive alterations in some models of convulsive epilepsy characterized by neurodegeneration, since LIR effects are likely secondary to its recognised neuroprotective properties.

Keywords: Epileptogenesis; GLP-1 receptor agonist; Kainate-induced seizures; Memory; WAG/Rij rats; Depression.

1. Introduction

Liraglutide (LIR) is a glucagon-like peptide-1 (GLP-1) receptor agonist currently approved for the treatment of type 2 diabetes mellitus (T2DM) (Peters, 2013) and under clinical evaluation for the treatment of Alzheimer's disease (Egefjord et al., 2012). GLP-1 is an endogenous incretin hormone released by the entero-endocrine L-cells in response to food ingestion with numerous effects on glycaemic homeostasis (Lovshin and Drucker, 2009). GLP-1 receptors (GLP1-Rs) are also expressed in the brain, particularly on pyramidal neurones in the hippocampus and neocortex, and Purkinje cells in the cerebellum (Hamilton and Hölscher, 2009). Furthermore, both astrocytes and microglia express GLP1-Rs being involved in the modulation of inflammatory responses (Iwai et al., 2006). Therefore, in addition to its metabolic effects, GLP-1 seems to act as a growth factor in the brain (Perry et al., 2007); in fact, GLP1-Rs in the hippocampus positively regulate neurite outgrowth, learning and long-term potentiation (LTP) (Abbas et al., 2009; Perry and Greig, 2005).

GLP1-R agonists have shown neuroprotective effects against glutamate-induced death in cultured hippocampal neurons (Perry, 2002) and in animal models of stroke (Hölscher, 2014). Several mechanisms have been proposed to contribute to the beneficial effects of GLP1-Rs on the brain including, reduced neuroinflammation and increased pro-survival cell signalling. GLP1-R agonists also enhance synaptic transmission, and counteract learning and memory deficits in different neurodegenerative conditions (During et al., 2003; Salcedo et al., 2012).

Novel GLP-1R agonists are likely to play a role in protecting neurons against several types of brain injuries, including excitotoxic and oxidative damage; in particular, LIR and lixisenatide cross the blood-brain barrier (BBB) when injected peripherally, and they increase the division of neuronal progenitor cells in the brain and enhance neurogenesis (Faivre et al., 2012; Hunter and Hölscher, 2012). LIR, a novel long-lasting GLP-1 analogue, also improves learning, memory and exerts neuroprotective effects in mouse models of Alzheimer's disease (Long-Smith et al., 2013; McClean and Hölscher, 2014), Parkinson's disease (Liu et al., 2015) and stroke (Sato et al., 2013). Thus, it is conceivable that these beneficial effects of GLP1-R (e.g. by LIR) activation may be relevant in other

brain diseases including epilepsy, where neuroinflammation is now recognized as an underlying contributory factor (Rana and Musto, 2018). Recently, it was demonstrated that LIR significantly decreased seizure severity (Koshal and Kumar, 2016a) and delayed seizure progression in a pentylenetetrazol (PTZ)-kindled mouse model (de Souza et al., 2019) and in the corneal kindling epilepsy model in mice (Koshal and Kumar, 2016b). The protective effects in these epilepsy models seem to be related to different mechanisms of LIR such as, positive regulation of hippocampal BDNF expression, reduction of oxidative stress (de Souza et al., 2019), prevention or reduction of pro-inflammatory cytokines and normalization of key neurotransmitters levels in the brain (Koshal and Kumar, 2016b, 2016a; Wang et al., 2018; Wen et al., 2019).

Epilepsy is a common neurologic disease and, at present, around 50 million people worldwide have active epilepsy with continuing seizures that need treatment, and ~30% of patients are resistant to conventional antiepileptic drugs (AEDs) (Laxer et al., 2014). Thus, development of new AEDs and new methods/approaches to treatment are urgently required. Furthermore, most evidence supports the view that development of epilepsy (epileptogenesis) is a process that should be treated as early and effectively as possible to try to prevent the development of chronic, intractable epilepsy; therefore, there is an urgent need to find ways to prevent the development of disease in individuals at risk (Jehi and Vezzani, 2014). Epileptogenesis refers to a dynamic process that progressively alters neuronal excitability, establishes critical (abnormal) synaptic interconnections, and perhaps requires intricate structural/biochemical changes before the first spontaneous seizure occurs (Pitkänen and Engel, 2014). In this light, the main objective of the present study was to investigate the effects of LIR on seizure development in two standard rodent models of epilepsy: a mouse model of convulsive temporal lobe epilepsy induced by kainate (KA) intra-hippocampal infusion (linked to hippocampal neurodegeneration) with co-morbid memory impairment and related anxiety-like behaviour, and a well-validated animal model of non-convulsive absence epilepsy and epileptogenesis, the WAG/Rij rat (not accompanied by neurodegenerative processes) with comorbid depressive-like behaviour (Russo et al., 2016a; Sarkisova and van Luijtelaar, 2011). Absence seizures are a type of non-

convulsive generalized epilepsy. They typically present with abrupt cessation of an ongoing activity, transient unresponsiveness to surrounding stimuli and generalized spike and wave complexes (SWDs) on EEG (Russo and Citraro, 2018). We also evaluated the effects of LIR on behavioural and cognitive alterations associated with epilepsy in both animal models, using some standard behavioural tests (Forced Swimming Test, Open Field Test and Morris Water Maze).

2. Materials and methods

2.1 Animals

Male C57BL/6 mice (6 weeks old) and male WAG/Rij rats (4 weeks of age) were purchased from Harlan Italy (Correzzana, Milan, Italy). Animals were housed three or four per cage and kept under controlled environmental conditions (60±5% humidity; 22 ± 2 °C; 12/12 h reversed light/dark cycle; lights on at 20.00). Animals were allowed free access to standard laboratory chow and water until the time of experiments. Animal care and experimental procedures were conducted in conformity with the international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration, including the "3R" concept). The experimental protocols and the procedures reported herein were approved by the Animal Care Committee of the University of Catanzaro, Italy. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Drugs

LIR (Victoza; Novo Nordisk S.p.A., Rome, Italy; pre-filled pen containing 18 mg liraglutide in 3 ml) was diluted in saline solution (0.9% NaCl) to obtain a final solution of 300 μg/ml and subcutaneously (s.c.) administered at a dose of 300 μg/kg/day in WAG/Rij rats. Instead for C57BL/6 mice, pre-filled pen containing LIR was diluted in saline solution (0.9% NaCl) to obtain a final solution of 300 μg/10 ml and administered s.c. at the dose of 300 μg/kg/day. Kainate (KA; Sigma Aldrich Milan, Italy) was

dissolved in saline (50 nl, 0.2 μg) and injected intrahippocampally (unilateral) at a volume of 0.5 μl (see below).

2.3 Unilateral intrahippocampal injection of KA and evaluation of LIR antiepileptogenic effects.

All male C57BL/6 mice were stereotaxically injected with KA into the CA1 area of the right dorsal hippocampus (coordinates from bregma: AP= −1.8 mm; L= −1.6 mm; and V= −1.5 mm according to Paxinos and Franklin atlas, 2001) under a mixture of tiletamine/zolazepam anaesthesia (1:1; Zoletil 100®; 25 mg/kg i.p.; VIRBAC Srl, Milan, Italy).

KA dissolved in saline (0.2 μg in 50 nl) was injected through a 0.5 μl microsyringe connected to a CMA/100 infusion pump over 60 s in order to induce *status epilepticus* (SE) which generally leads to the development of spontaneous seizures with hippocampal neurodegeneration within 1 month (Citraro et al., 2015a; Gröticke et al., 2008). Control mice received an identical volume of saline instead of KA solution. After injection, the needle of the syringe was maintained *in situ* for an additional 2 min. None of the animals died during the surgical procedure or after KA infusion.

KA-treated mice were monitored by simultaneous EEG/video recording for detection of spontaneous seizures (SRS) induced by KA after 4 weeks (see below). These observations were done supporting the hypothesis that following intrahippocampal KA injection in male C57BL/6 mice, there is a latent period of 5-7 days between the initial neuronal injury associated with KA treatment and the occurrence of spontaneous electrographic seizures associated with chronic epileptogenesis. Therefore, mice were treated with LIR starting 6 h after KA injection.

Mice were then assigned to 4 groups: 1) Control vehicle-treated mice (n = 20); 2) Control LIR-treated mice (n = 20); 3) KA vehicle-treated mice (n = 20); 4) KA LIR-treated mice (n = 20).

LIR (300 μg/kg s.c.) was administered once a day for 4 consecutive weeks between 9 and 10 am and then suspended in order to perform EEG/video recordings in drug free animals in order to avoid any potential direct effect on SRS by LIR. Control mice (receiving vehicle) were daily s.c. injected with saline solution (0.9% NaCl).

For EEG/video recordings, about the beginning of the third week, all animal groups were bilaterally implanted under anaesthesia (see below) with four stainless steel electrodes fixed in the skull over the frontal and the parietal cortex. Two other electrodes were placed bilaterally over the cerebellum to serve as ground and reference electrodes. Implantation of electrodes was done to monitor any end modification on the development of spontaneous seizures throughout the epileptogenic period. EEG and video recordings of freely moving mice placed in Plexiglass test cages were obtained using a Pinnacle Technology's 8400–9000 video/EEG system with Sirenia Software, Kansas, (USA) as below described. For detection of KA-induced spontaneous seizures, the EEG/video recordings were visually analysed for characteristic paroxysmal (epileptiform) activity. Furthermore, the detection of at least one SRS present in the EEG and interictal spiking but usually not accompanied by obvious behavioural correlates (defined by video recording) was used as a criterion to judge the animal as epileptic. Control mice with implanted electrodes were monitored by EEG/video recording in order to evaluate whether seizure-like events were also present in controls, *e.g.* induced by depth electrode implantation. EEG/video recordings were performed for 3 hours in the morning between 9 and 12 in two consecutive days on week 4, starting 1 day after drug suspension (Klein et al., 2015).

For the determination of hippocampal damage, animals were anesthetized as above reported, and killed by transcardiac perfusion with cold phosphate buffer saline (PBS), pH 7.4 and subsequently with cold 4% paraformaldehyde, containing 0.2% saturated picric acid in PBS. Brains were removed, post-fixed overnight at 4°C in the same fixative solution. Paraffin embedded sections were then cut in a coronal plane at a thickness of 4 μm by a microtome. The de-paraffined sections were rinsed in PBS twice and immersed in 0.3% H_2O_2 in PBS for 10 min followed by three rinses in PBS.

Paraffin-embedded brain sections were de-paraffined with xylene and rehydrated with ethanol at graded concentrations of 100–70% (v/v), followed by washing with distilled water. Sections were stained with 0.1% (w/v) cresyl violet and severity of neuronal damage was evaluated by the number of surviving neurons (*i.e.* conventionally intact cells when stained with cresyl violet) in the hippocampus. Briefly, the mean number of surviving neurons per 100 μm length was separately

calculated in the CA1 hippocampal sector to accurately estimate the extent of neuronal damage. Cell counting was performed in six serial sections per mouse in a blind fashion by two independent observers, using a light microscope equipped with a 20x objective (Palleria et al., 2017).

2.4 Experimental protocol in WAG/Rij rats

LIR was administered subcutaneously at a dose of 300 µg/kg/day (between 9 and 10 am) that is considered the maximum tolerated dose in rodents, since higher doses induce significant weight loss and are not tolerated in the long term (Hendarto et al., 2012; Palleria et al., 2017).

LIR (300 µg/kg/day) treatment was started in WAG/Rij rats at 30 days of age (n = 10) and continued for a further 17 weeks until the age of ~5 months, when it was stopped, as previously described (Citraro et al., 2017; Russo et al., 2013c). Thereafter, the animals continued to be normally housed without treatment under standard conditions. Age-matched control rats (*n* = 10 WAG/Rij rats) were kept under the same housing conditions over the same time window. During this period, animals were weekly weighed, every Monday, between 9:00 and 11:00 a.m.

2.5 Surgery and EEG recordings in WAG/Rij rats

WAG/Rij rats around the age of 6 months, 30 days after treatment discontinuation, were chronically implanted, under anaesthesia obtained by administration of a mixture of tiletamine/zolazepam (1:1; Zoletil 100®; 50 mg/kg i.p.; VIRBAC Srl, Milan, Italy), using a Kopf stereotaxic instrument, with 2 cortical electrodes for EEG recordings and the ground electrode placed over the cerebellum as previously described (Citraro et al., 2015b; Leo et al., 2017). At the end of at least 1 week of recovery, EEG recordings were performed to evaluate the effects of drug on spontaneous spike wave discharges (SWDs). The animals were attached to a multichannel amplifier (Pinnacle Technology's 8400–9000 video/EEG system with Sirenia Software, Kansas, USA) by a flexible recording cable and an electric swivel, fixed above the cages, permitting free movements for the animals. All EEG signals were amplified and conditioned by analog filters (filtering: below 1 Hz and above 30 Hz at 6 dB/octave)

and subjected to an analog-to-digital conversion with a sampling rate of 300 Hz. All WAG/Rij rats (treated and untreated) underwent three recording periods, for 3 consecutive days, as previously described (Russo et al., 2013b). Every recording session lasted 3h without administration of any drug in either group. The blinded quantification of absence seizures was based on the number and the duration of EEG SWDs, as previously described (Citraro et al., 2015c; Leo et al., 2017).

2.6 Behavioural tests

Every experimental group of rats and mice underwent the following tests in order to evaluate the development of behavioural alterations and/or drug effects (LIR was suspended on the last day of the 4th week and tests were performed in order to measure chronic effects): forced swimming test (FST); sucrose preference test, Open field (OF); Morris Water Maze (MWM) and Rota-rod test. All behavioural tests were carried out under controlled conditions of temperature and humidity; light intensity was dependent on the respective experimental setup (Russo et al., 2016b, 2013a, 2013b). All tests were carried out with the support of EthoVision XT8 Software from Noldus (Netherlands).

2.6.1 Forced swimming test (FST)

The forced swimming test (FST) has been previously used for measuring the immobility time (IT) and assess depressive-like behaviour in rodents (Sarkisova and Petrzik, 2011). The FST was used to reveal behavioural differences related to depressive-like state in rats or mice treated with LIR. Briefly, rats or mice were individually forced to swim during 6 min in a clear plastic cylinder (47 cm in height; 38 cm in diameter for rats; height: 26.5 cm, diameter: 16.5 cm for mice) containing 38 cm (for rats) or 15 cm (for mice) of water maintained at 22–23 °C. The total duration of immobilization, including passive swimming, was measured in the last 4 minutes of the test. The criterion for passive swimming was floating vertically in the water while making only those movements necessary to keep the head above the water. After the FST, animals were removed and dried with a towel before being placed in their home cages. Every experimental animal group was evaluated in the test always between 9.00

a.m. and 11.00 a.m. in order to avoid possible circadian alteration of test results (Citraro et al., 2017; Russo et al., 2013a).

2.6.2 Sucrose preference test (SPT)

Anhedonia is a key component of depression and can be measured in mice by their preference to consume a sweetened solution. For the sucrose preference test, mice were placed in separate cages and habituated to a 2% sucrose solution for 48 hours before the test day. Mice were deprived of water and food for the 4 hours from 8:00 pm to 12:00 pm and this was followed by a 1-hour preference test with water and 2% sucrose delivered from identical bottles. The bottles containing water and sucrose were weighed before and after this period, and sucrose preference (%) was determined. The sum of the water plus sucrose intake was defined as the total intake, and a sucrose preference was expressed as the percentage of sucrose intake relative to the total intake (Russo et al., 2013a).

2.6.3 Open-field test (OF)

Locomotor activity was monitored for 10 min in an open field, a white Plexiglas box 100 x 100 cm (for rats) or 50×50 cm (for mice) with its floor divided into 32 (for rats) or 16 (for mice) squares. Four squares were defined as the center of the field. Each animal was gently placed in the center of the box, and activity was scored, as previously described (Russo et al., 2013b, 2013a). After each trial, the chamber was cleaned with water containing an odourless detergent. The following parameters were considered: the time spent in the center, the total distance moved, and the mean velocity during the 10-min test. An increase of time spent in the center or a decrease of the latency to enter the center are indicators of anxiolytic activity and vice versa (Prut and Belzung, 2003).

2.6.4 Morris water maze test (MWM)

Learning and memory functions were assessed using a spatial acquisition task in a Morris water maze (MWM) test, as previously described (Palleria et al., 2017; Russo et al., 2013a). The apparatus

consisted of a circular basin (diameter =180 cm, height = 50 cm for rats; diameter = 93 cm, height = 45 cm for mice) filled with water (approximately 25 °C) to a depth of 30 cm (for rats) or 24 cm (for mice), with a clear (invisible) escape platform (diameter =8 cm) placed 1 cm below the water surface. Several visual cues surrounding the maze were available on the walls, and the observer remained in the same location for each trial. The position of the platform and the cues remained the same throughout the whole experiment. Animals (n=10 from each group) were trained for 4 consecutive days, with 4 trials on each day. During each trial, individual animals were put in a randomly chosen quadrant in the pool with the head facing the pool wall. Each animal was given 60 seconds (s) to search for and locate the submerged platform. If an animal failed to locate the platform within 60 s, it would be guided gently to the platform. The latency time to find the platform was recorded, and the average time from 4 trials represented the daily result for the animal. On the 5th day of the MWM test, each animal was subjected to a probe test where no platform was present. The time of swimming in the former platform quadrant and the total time of swimming in all four quadrants were recorded for 60s. The percentage of swimming in the quadrant of the former platform was calculated as a measurement of spatial memory (Russo et al., 2013a).

2.6.5 Rotarod test

The rotarod test (LE 8500 RotaRod for 4 rats or 4 mice with Software; 2biological instruments; Besozzo VA Italy) was used to assess motor coordination by testing the ability of animals to remain on a revolving rod. The apparatus consisted of a cylinder of 3 cm of diameter rotating at 5 rpm simulating a treadmill test. Before the training session, mice were habituated for two consecutive days to stay on the rotating rod. On the third day, the two animal groups (control group and LIR treated-group) were tested taking the time spent by the animals on the rotarod for a period 5 min. The animals were placed on the rotating rod with head directed against the direction of the rotation so that the animals had to progress forward to maintain equilibrium. Motor integrity and coordination were

assessed by the time latency from placement of the animal on the rotating drum until it fell. Latency times are measured in seconds (Palleria et al., 2017).

2.7 Measurement of blood glucose levels

Blood samples in each group were obtained from the caudal vein at the same time of day (10:00 am-11:00 am) once a week for 17 weeks of treatment in rats and once a week for 4 consecutive weeks of treatment in mice. Blood glucose was measured by using a blood glucose meter, ONETOUCH Ultra Vue (Johnson & Johnson Corp., New Brunswick, NJ, USA).

2.8 Statistical analysis

All statistical procedures were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA). EEG recordings were subdivided into 30 min epochs, and the duration and number of SWDs were treated separately for every epoch. Such values were averaged and data obtained were expressed as mean ± S.E.M. EEG data were analysed and compared by 1-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test.

Data obtained by behavioural tests and Western blotting analysis from the long-term treatment schedule were analysed and compared by 1-way ANOVA followed by Bonferroni's post-hoc test. Data obtained by Western blotting analysis from treatment were analysed and compared by the Student's t-test. All tests used were 2 sided and $P \leq 0.05$ was considered significant.

3. Results

3.1 Effects of LIR in the intrahippocampal KA-induced mouse model

3.1.1 LIR effects on the development of SRS induced by unilateral intrahippocampal KA infusion in mice

Single unilateral injection of KA into the hippocampus of mice induces an initial SE which initiated epileptogenesis and the development of SRS after a latent period (5–21 days) (Jefferys et al., 2016;

Lévesque and Avoli, 2013). Mice of all groups were video/EEG recorded at 4 weeks post-KA injection, during the chronic phase of the disease, when recurrent seizures could be observed and starting 1 day after drug suspension in order to avoid measuring any potential direct drug effect on SRS but only its antiepileptogenic effects.

In the KA vehicle-treated mice, EEG recordings, 4 weeks after SE, revealed recurrent SRS in the cortex, especially on the ipsilateral side to the KA injection, correlated with the chronic phase (Fig. 1A). These SRS, (3–7s), occurred either without any perceptible clinical manifestations or with immobility, staring and behavioural arrest, sometimes associated with facial movements. Rare motor seizures were observed in KA-treated mice. When occurring, they were characterized by behavioural arrest, vocalization, mastication, clonic movements of the forepaws and rearing. For the entire duration of the recording, SRS were more frequent in the KA injection side as compared to the contralateral one. The number and total duration of SRS and seizures detected during EEG recording are presented in (Fig. 1). By contrast, no EEG alterations were observed in control vehicle-treated mice (Fig. 1B, C). In KA LIR-treated mice, administration of the GLP-1R agonist at a dose of 300 µg/kg/day s.c. significantly decreased ($P< 0.05$) the number and total duration of SRSs (spontaneous recurrent seizures) compared with KA vehicle-treated mice, reducing the epileptogenic process (Fig. 1B, C).

3.1.2 LIR effects on behavioural alterations in the intrahippocampal KA-induced mouse model

In contrast to WAG/Rij rats, statistical analysis indicated that LIR treatment significantly ($P < 0.05$) increased IT in Control LIR-treated and KA-LIR-treated groups in the FST (Fig. 2A). In addition, LIR treatment significantly reduced ($P < 0.05$) both mean velocity and total distance moved in Control LIR-treated and KA LIR-treated groups, suggesting a pro-depressive effect (Fig. 2B, C). We measured the velocity and total distance moved every minute for four minutes of the FST. In the first three minutes there was no significant difference in velocity and distance moved between the experimental groups while in the last minute of the FST, LIR-treated mice showed less velocity and

distance moved compared to untreated groups suggesting that treatment with LIR might in fact, induce fatigue in mice rather than depression. Considering this latter possibility, in order to better evaluate LIR effects on depressive-like behaviour, we next addressed the issue of anhedonia by examining whether preference of a sucrose solution would differ between LIR-treated mice and control mice. Statistical analysis however indicated that there was no decrease in sucrose intake for the LIR-treated group ($P > 0.05$) when compared to control group. Water intake also did not differ significantly between the groups. The LIR-treated mice did not show any effect on the percentage of sucrose preference when compared to control mice, thus indicating the absence of LIR effects on depression-like behaviour (see Supplementary File Fig. S1).

Finally, in support of the "fatigue" hypothesis, we found that LIR-treated mice spent less time walking on the rotarod in comparison to control mice. LIR-treated mice walked for a significantly ($P<0.05$) shorter time on the rotarod (848 ± 414.2 s) compared to control mice (2074.3 ± 371.6 s). Therefore, the increase of IT observed in the FST did not reflect a depressive-like behaviour, but rather a state of fatigue of LIR-treated mice as shown in the rotarod test (Fig. 2D).

The results obtained in the OF test revealed, in KA vehicle-treated group, that the time spent and the number of entries in the center, which are inversely correlated to the level of anxiety/emotionality in mice (Prut and Belzung, 2003), were decreased in comparison to the control vehicle-treated group indicating an increased anxiety following KA administration (Fig. 3A, B). LIR treatment significantly increased ($P<0.05$) the time spent in the center in Control LIR-treated and KA LIR-treated groups without modifying the number of entries in the center (Fig. 4A, B). Mean velocity and total distance moved did not significantly differ between experimental groups (see Supplementary File Fig. S2).

Memory/cognitive functions were tested in the MWM; we found that the KA-vehicle group showed a significant impairment in learning and memory ($P<0.01$) with a longer latency to find the platform during daily trials starting from day 2 (Fig. 4A). Furthermore, as shown in Fig. 4B, KA significantly reduced ($P < 0.01$) the time spent in the former platform quadrant on day 5. LIR treatment reduced the latency to platform already on day 1 indicating a favourable effect on learning in mice with

memory impairment (KA group) (Fig. 4A). Furthermore, LIR significantly increased the time spent in the former platform quadrant on day 5 (Fig. 4B). No effects were evident in Control LIR-treated mice compared to untreated animals (Control vehicle-treated mice). Regarding locomotor activity, considering mean velocity and total distance moved, no significant differences were noted between treated and untreated groups (P>0.5).

3.1.3 Effects of LIR on histopathological alterations in KA-induced SE

Control vehicle-treated mice, which received intrahippocampal injection of saline and were implanted with an EEG cortical electrode, did not exhibit degeneration of hippocampal neurons (Fig. 5A). However, administration of KA led to the degeneration and a statistically significant neuronal loss of CA1 pyramidal cells in comparison to the control group (Fig. 5A, B) in accordance with other studies (Rattka et al., 2013). We observed also that occasionally, the neuronal damage expanded into other hippocampal subsectors. Treatment with LIR reduced the KA-triggered neuronal loss in the CA1 subsector of the hippocampus (Fig. 5C, D). Therefore, statistically significant (P = 0.037) neuroprotective effects of LIR were observed as compared to KA vehicle-treated mice (Fig. 5D).

3.2 Effects of LIR treatment in WAG/Rij rats

3.2.1 Effects of early long-term treatment with LIR on absence seizures in WAG/Rij rats

In control WAG/Rij rats at 6 months of age, the mean number of SWDs (nSWDs) per 30-min epoch was 7.63 ± 0.51, mean total duration of SWDs (dSWDs) was 25.49 ± 2.24 s and mean single SWD duration (sSWD) was 3.25 ± 2.39 s.

Early long-term treatment with LIR at a dose of 300 μg/kg/day s.c. was unable to significantly (P > 0.05) reduce the development of absence seizures in adult WAG/Rij rats, compared to untreated controls. All parameters of SWDs were not significantly (P > 0.05) modified. Animal growth was very similar within the groups, with no significant differences between control and LIR-treated animals over the 17-week treatment period (see Supplementary File Fig. S3 and S4).

3.2.2 LIR effects on behaviour in WAG/Rij rats

We also examined the effects of long-term treatment with LIR on anxiety and depressive-like behaviour in WAG/Rij rats at 6 months of age. WAG/Rij rats showed depressive-like behaviour according to an increased IT in the FST, as previously described (Mattace Raso et al., 2013; Sarkisova et al., 2010). Early long-term treatment with LIR, significantly ($P < 0.034$; about 55%) reduced the IT (Fig. 6), therefore decreasing depressive-like behaviour; the total distance moved and mean velocity were not however, significantly different (see Supplementary File Fig. S5). On the other hand, LIR had no effects in WAG/Rij rats in comparison to control untreated group when animals were tested both in the OF and MWM tests (see Supplementary File Fig. S6 and S7).

3.3 Effects of LIR treatment on blood glucose level

Since LIR is clinically used as an antidiabetic drug (Peters, 2013), we also monitored blood glucose levels over the 17 and 4 weeks of LIR treatment in WAG/Rij rats and in mice with KA-induced SE respectively. No significant differences in blood glucose levels between the LIR and control-treated animals were observed in either group (see Supplementary File Table S1 and S2).

4. Discussion

In the present study, we have investigated the possible effects of LIR, a GLP-1 analogue used in T2DM treatment, on epileptogenesis and related neuropsychiatric comorbidities in two different animal models of epilepsy. Similarly to the native GLP-1, LIR crosses the BBB and is able to bind to GLP-1Rs in the brain, exerting neuroprotective effects (Candeias, 2015; Hunter and Hölscher, 2012). Accordingly, several preclinical studies have demonstrated anti-apoptotic, anti-inflammatory, anti-oxidant and neuroprotective effects of LIR against stroke (Sato et al., 2013), Parkinson's disease (Liu et al., 2015) and Alzheimer's disease models (Long-Smith et al., 2013; McClean and Hölscher, 2014).

Effects of LIR on epileptogenesis in WAG/Rij and kainate model

In the present study, LIR was completely ineffective in preventing the appearance and development of absence seizures in WAG/Rij rats, a genetic animal model of absence epilepsy, epileptogenesis, and mild-depression comorbidity while it was effective in preventing the development of spontaneous seizures in the intrahippocampal KA SE animal model.

The different effect of LIR against epileptogenesis in the two epilepsy models, could easily be attributable to the nature of the epileptogenic process in the two cases. In the KA model, hippocampal neurodegeneration is at the basis of seizure development (Arabadzisz et al., 2005; Riban et al., 2002) and LIR, being neuroprotective, in reducing CA1 neuronal degeneration among other potential mechanisms also prevented the appearance of spontaneous seizures while, in WAG/Rij rats, no neurodegeneration is generally observed and the development of spontaneous absence seizures is likely based on genetically driven network adaptations (Russo et al., 2016a). Therefore, the selective effect of LIR may depend on its ability to interact with specific neurodegenerative mechanisms which are not present in the genetic absence epilepsy model. Indeed, the differential effect could be based on a selectivity of action on seizure type or even the brain areas involved; the hippocampus is not fundamentally involved in absence seizures and LIR may act specifically on the hippocampus which is widely expressing GLP-1 receptors (Cork et al., 2015; Jensen et al., 2018).

The antiepileptogenic effects of LIR in the KA model on the other hand, are most likely due to the neuroprotective effect on CA1 neurones and this latter effect could be due to the known LIR anti-inflammatory effects (Parthsarathy and Hölscher, 2013; Shiraki et al., 2012). Intrahippocampal injection of the KA in mice induces a limbic SE and a characteristic pattern of neuronal degeneration in the CA1 area of the hippocampus (Bouilleret et al., 1999; Gröticke et al., 2008), resembling the characteristic hippocampal alterations in patients with mesial temporal lobe epilepsy (TLE) (Malmgren and Thom, 2012).

LIR effects on the inflammatory pathway and cell death could contribute to the neuroprotective effects of this drug as also previously demonstrated in other epilepsy models (Koshal and Kumar,

2016a; Wang et al., 2018). However, this may not be the case for WAG/Rij rats in which it is known that increasing inflammation by LPS administration is pro-epileptic through cytokine release (Kovács et al., 2011, 2006; Russo et al., 2014) and that some drugs with anti-inflammatory effects reduce absence seizures (Citraro et al., 2015c; Kovács et al., 2014; Rimoli et al., 2009). In fact, it was previously confirmed that inflammatory cytokines may have a role in absence seizures in this strain as well as in GAERS (Akin et al., 2011; Györffy et al., 2014; Van Luijtelaar et al., 2012); however, they do not seem to be involved in the epileptogenic process (Russo et al., 2016a).

In agreement with our findings, LIR has been demonstrated to prevent the development of seizure severity in corneal kindling epilepsy and to improve its related secondary complication (such as depression and cognitive impairment), and neurochemical alteration in mice (Koshal and Kumar, 2016b). Also, it has been shown that GLP-1R deficient mice have an enhanced seizure severity and neuronal injury after KA administration, with correction after GLP-1R gene transfer in hippocampal somatic cells (During et al., 2003). The mechanisms through which this increase in cell viability occurs could include activation of the transcription factor p90RSK, which modulates the expression of genes associated with the cellular response to stress (Sharma et al., 2014). It has also been suggested that LIR-mediated neuroprotection may involve the PI3K/Akt pathway and its subsequent regulation of mammalian target of rapamycin (mTOR) (Kimura et al., 2009). A recent study in the lithium-pilocarpine-induced SE model has demonstrated that LIR reduced the chronic inflammation and mitochondrial stress thereby exerting neuroprotective effects (Wang et al., 2018). In PTZ-induced kindling, LIR delayed the full kindling development and anxiety- and depressive-like behaviours and cognitive dysfunction. The mechanism by which LIR prevented these alterations may involve the positive regulation of BDNF expression in the hippocampus and the reduction of oxidative stress (de Souza et al., 2019). Very recently, it was shown that activated GLP-1R can modulate the excitability of the CNS and its neuronal expression levels are modified accordingly; GLP-1R levels were reduced in the PTZ-treated rats as well as TLE patients. Also LIR treatment significantly up-regulated GLP-1R and GABAARb2/3 and down-regulated GluA1-4, GluNR1, GluN2A and GluN2B exerting

antiepileptic effects in epileptic rats (Wen et al., 2019). Therefore, several mechanisms may account for the LIR antiepileptogenic and neuroprotective effects in the KA model.

Effects of LIR on animal behaviour in WAG/Rij and kainate model

Regarding animal behaviour (neuropsychiatric comorbidities), different results were found in the two models. In fact, LIR reduced IT for WAG/Rij rats, showing antidepressant effects, and increased IT in KA model during the FST. The selective action of LIR, on the hippocampus, as previous described, could explain the observed LIR effect on depressive-like behaviour in WAG/Rij rats; in fact, LIR antidepressant-like effects were also previously reported in an animal model of depression induced by long-term antipsychotic treatment in rats (Sharma et al., 2014). The mechanism underlying this antidepressant effect however, remains unclear. Instead the increased IT in KA model during the FST was justified by the fact that a motor impairment was observed confirming our previous results in rats (Palleria et al., 2017). Therefore, this increase in IT in the KA model is apparently due to an LIR-induced state of fatigue or reduced resistance to stress, and not to a real pro-depressant effect. However, it remains to be determined why a similar effect was not observed in the WAG/Rij rats; a reasonable explanation may be the fact that KA-treated mice have a normal IT while WAG/Rij rats present with an increased IT suggestive of an intrinsic depressive-like state, therefore, LIR may rescue this behaviour and the final result is the balance between a direct antidepressant effect and its negative effects on locomotor activity.

Similarly, LIR increased the time spent in the center in the OF test, indicating potential anxiolytic effects and prevented memory/learning decline in KA vehicle-treated mice, thereby improving mouse performance in the MWM, while it had no effects in the same tests in WAG/Rij rats.

The observed difference between the two models in the MWM test can also be explained by the fact that WAG/Rij rats at 6 months of age do not have an altered memory/learning performance when measured in the MWM (Karson et al., 2012) while KA-treated mice have an impairment which is probably linked to the hippocampal degeneration; therefore, selective effects could again be

explained by LIR-neuroprotective effects more than a direct nootropic effect. Accordingly, GLP-1 and GLP-1Rs are highly expressed in the brain, including the hippocampus, a brain area involved in memory formation (During et al., 2003; Hamilton and Hölscher, 2009). Overexpressing GLP-1Rs in the hippocampus increased neurite growth and improved learning and memory in the MWM (During et al., 2003), whereas GLP-1R knockout mice had an impaired development of hippocampal long-term potentiation (LTP) and spatial learning and memory (Abbas et al., 2009). One of the possible mechanisms by which LIR prevents cognitive deficits may be the upregulation of intracellular cAMP levels (Han et al., 2013; Hunter and Hölscher, 2012). Unexpected was the different outcomes in the OF test; LIR did not apparently have any effect in WAG/Rij rats while increasing the time spent in the center in mice independently of KA treatment; therefore, this anxiolytic effect appeared to be independent from seizures, as also previously described (Palleria et al., 2017). This differential result is probably based on genetic differences between the two animal strains and deserves further investigation to determine whether there is any specific explanation and mechanism or even determine whether a real anxiolytic effect is possessed by LIR.

4.1 Conclusions

In conclusion, we have demonstrated for the first time, that treatment with LIR has antiepileptogenic and neuroprotective properties in the unilateral intrahippocampal KA model of temporal lobe epilepsy; furthermore, this effect seemed to be specifically linked to the ability of the drug to protect the brain from neurodegeneration while it may not be effective against epileptogenic processes different from those related to brain damage such as in the case of genetically determined absence epileptogenesis (Russo et al., 2016a). Furthermore, LIR seems to have a positive effect on epilepsy comorbidities and more specifically, on cognitive functions, anxiety and depression. However, also in this case, LIR effects may be limited only to some epilepsy models and therefore some selected cases. Finally, the antiepileptogenic and positive behavioural effects of LIR (and possibly other GLP1-R agonists) warrant further confirmatory studies in other animal models as well as human

trials, and some information is also needed from clinical studies above all in the areas of mood, anxiety and cognition. The future use of GLP-1R agonists for the general treatment of convulsive epilepsy either alone or in combination with existing AEDs could thus add a new and long-awaited dimension to the treatment of this chronic and debilitating condition worldwide.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1 Isolated spikes in the video/EEG recording observed in KA-treated mice **(a).** LIR significantly reduced (*P <0.05) the number **(b)** and duration **(c)** of KA-induced seizures measured over a time period of 30 min epochs. The values were averaged from 6 hours recordings obtained from two recording session of 3h each on two consecutive days 4 weeks after KA administration and 1 day after LIR treatment suspension; data obtained were expressed as mean ± S.E.M. *KA= Kainate; LIR= Liraglutide*.

Fig. 2 Forced swimming test (FST) and Rotarod test in KA-induced epilepsy. Effects of LIR treatment on immobility time (IT; expressed in seconds) **(a)**, on distance moved (expressed in cm) **(b)** and on mean velocity (expressed in cm/s) **(c)** FST**.** The influence of LIR on the latency to fall in the rotarod test **(d).** Values are means ± S.E.M.; Data marked with (*) are significantly different (P<0.05) from untreated mice. *CTRL =Control; CTRL-LIR= Control+Liraglutide; KA= Kainate; KA-LIR= Kainate+Liraglutide.*

Fig. 3 Open field (OF) results in KA-induced epilepsy. Effects of LIR treatment on anxiety level, in KA-induced epilepsy, measured by time spent in the central zone (sec) *vs* total time spent in the OF **(a),** and number of entries in the center **(b).** Values are mean ± S.E.M. Data marked with (#) are significantly different (P < 0.01) from CTRL animals. (*) Significantly different (P< 0.05) from untreated LIR group. *CTRL =Control; CTRL-LIR= Control+Liraglutide; KA= Kainate; KA-LIR= Kainate+Liraglutide.*

Fig. 4 Morris water maze (MWM) results in KA-induced epilepsy. Learning curve (latency time to reach platform) over 4 consecutive days in MWM **(a);** time spent in the target quadrant during the probe test on the fifth day **(b)**. (*) Significantly different (P< 0.05) from untreated LIR group. *CTRL =Control; CTRL-LIR= Control+Liraglutide; KA= Kainate; KA-LIR= Kainate+Liraglutide.*

Fig. 5 Histological analysis of LIR effects on neuronal injury induced by KA injection in mice. Cresyl violet staining was performed on sections from the hippocampal CA1 region. CTRL mice show large conical shaped pyramidal neurons, with well demarcated amphophillic cytoplasm and round vesicular nuclei with prominent nucleoli, and no signs of degeneration **(a);** CA1 region of mice following KA-injection shows distinct neuronal alterations characterized by pronounced shrinkage of the neuronal bodies with loss of nuclei and pyknotic pyramidal cells **(b)**; CA1 region of KA-injected mice and subsequent treatment with LIR shows some protection against KA -induced cell loss and pyknotic cells **(c);** LIR treatment significantly prevented neuronal cell loss in the hippocampal CA1 region in KA-treated mice **(d).** Values are means ± S.E.M.; data marked with (*) are significantly different ($P < 0.05$) from CTRL animals; # Significantly different ($P< 0.05$) from KA-treated group. *CTRL =Control; CTRL-LIR= Control+Liraglutide; KA= Kainate; KA-LIR= Kainate+Liraglutide.*

Fig. 6 Forced swimming test (FST) in WAG/Rij rats. Bars indicate the immobility time (IT), expressed in seconds, in the FST in WAG/Rij rats at 6 months of age following an early long-term treatment with LIR at 300 μg/kg/day (started at P30 and lasting 17 weeks). LIR significantly reduced the IT (*P <0.05) compared with age-matched control rats (CTRL). *CTRL=Control; LIR= Liraglutide.*

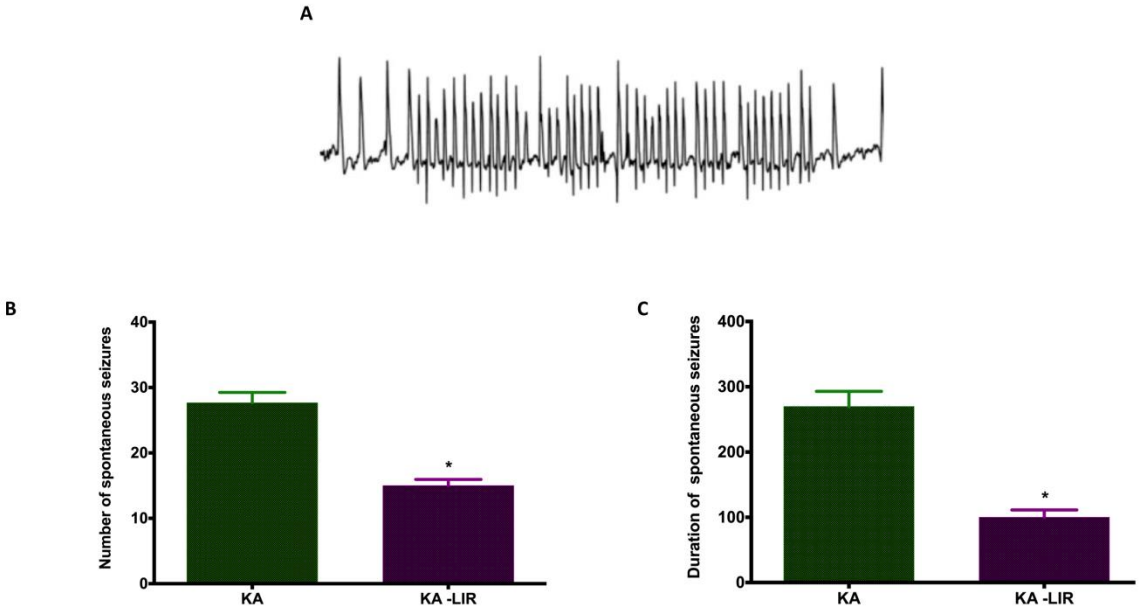
A
B
Number of spontaneous seizures
40
30
20
10
0
*
KA
KA -LIR
C
Duration of spontaneous seizures
400
300
200
100
0
*
KA
KA -LIR

Fig.1

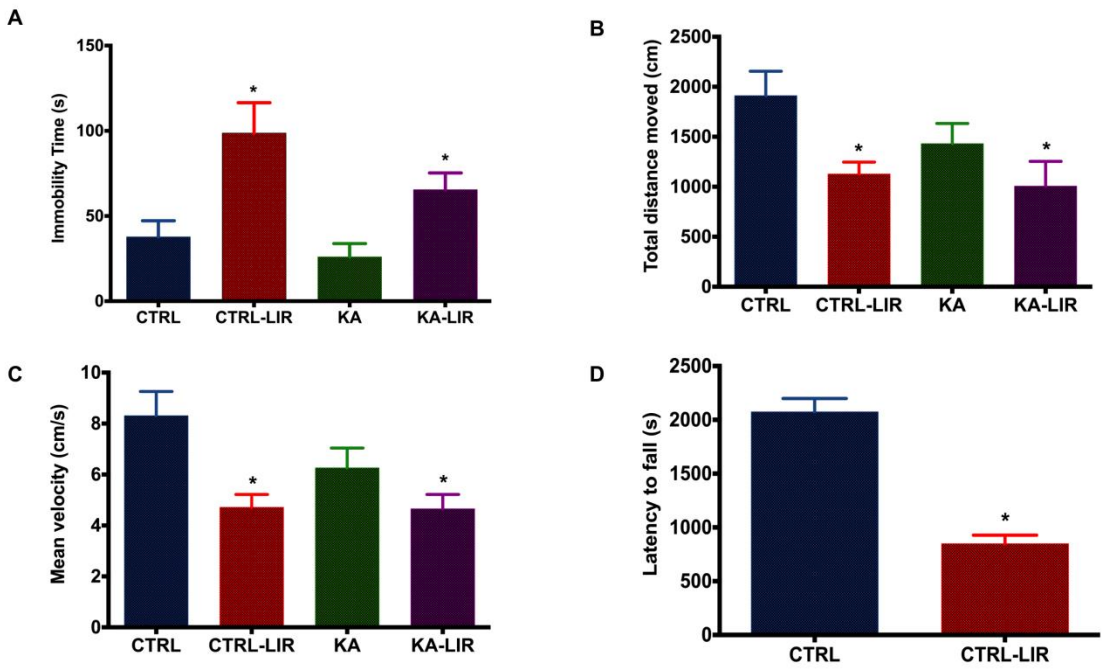
A
Immobility Time (s)
150
100
50
0
CTRL
CTRL-LIR
KA
KA-LIR
B
Total distance moved (cm)
2500
2000
1500
1000
500
0
CTRL
CTRL-LIR
KA
KA-LIR
C
Mean velocity (cm/s)
10
8
6
4
2
0
CTRL
CTRL-LIR
KA
KA-LIR
D
Latency to fall (s)
2500
2000
1500
1000
500
0
CTRL
CTRL-LIR

Fig.2

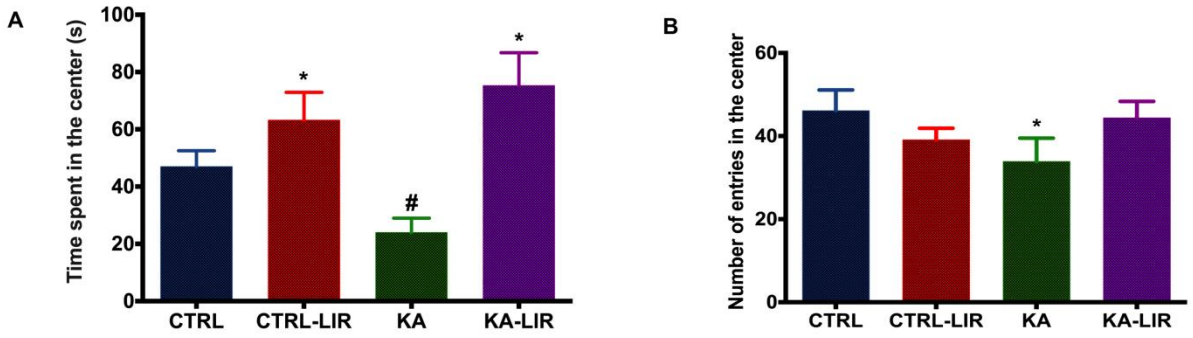
A
Time spent in the center (s)
100
80
60
40
20
0
*
#
*
CTRL
CTRL-LIR
KA
KA-LIR
B
Number of entries in the center
60
40
20
0
*
CTRL
CTRL-LIR
KA
KA-LIR

Fig.3

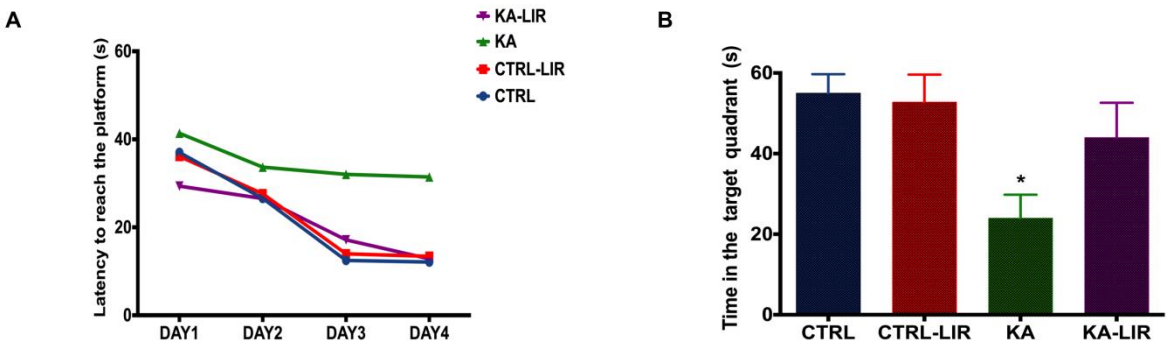
A
KA-LIR
KA
CTRL-LIR
CTRL
Latency to reach the platform (s)
60
40
20
0
DAY1
DAY2
DAY3
DAY4
B
Time in the target quadrant (s)
60
40
20
0
*
CTRL
CTRL-LIR
KA
KA-LIR

Fig 4

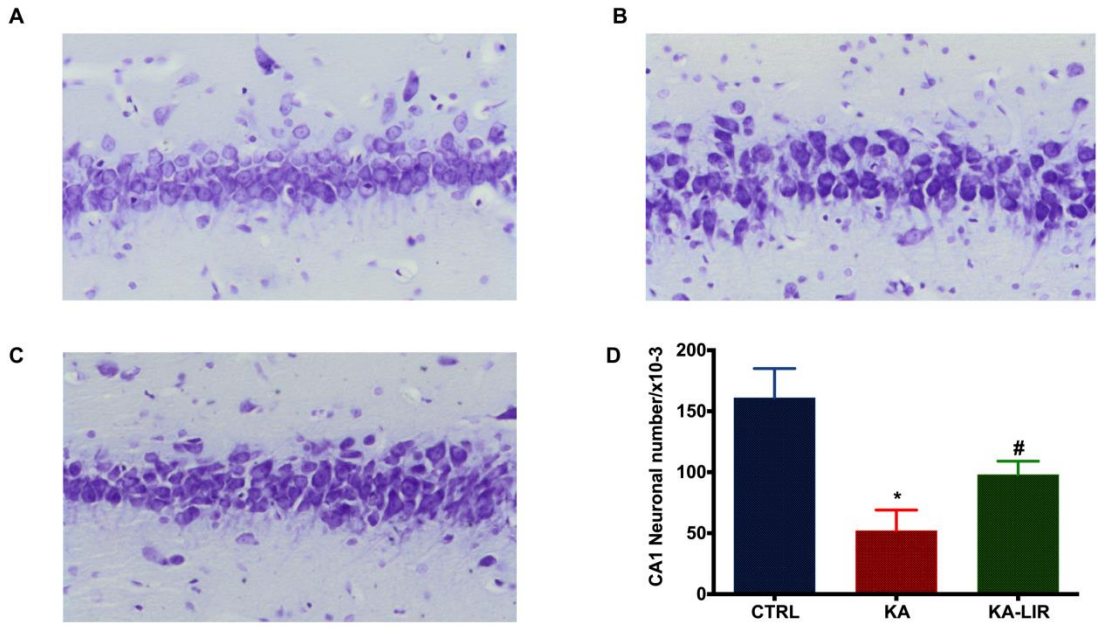

Fig.5

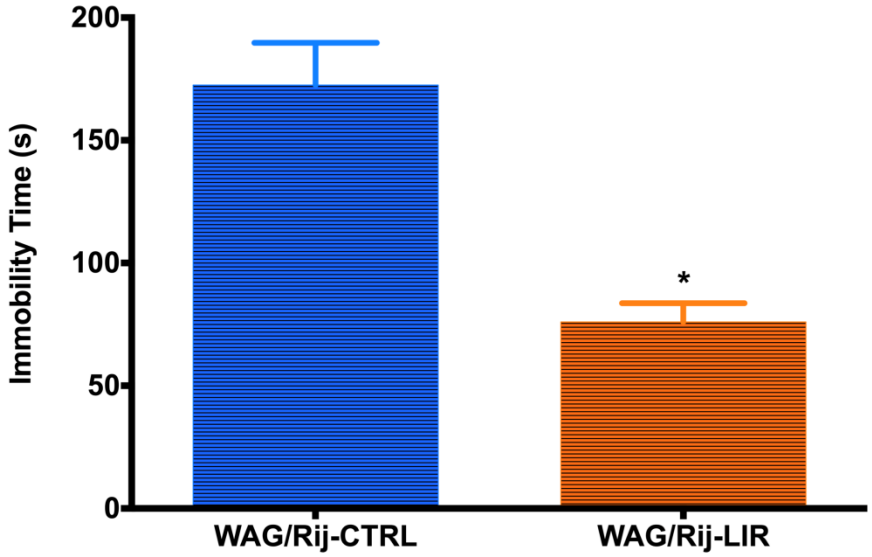

Fig. 6

1. Effects of LIR treatment on kainate model

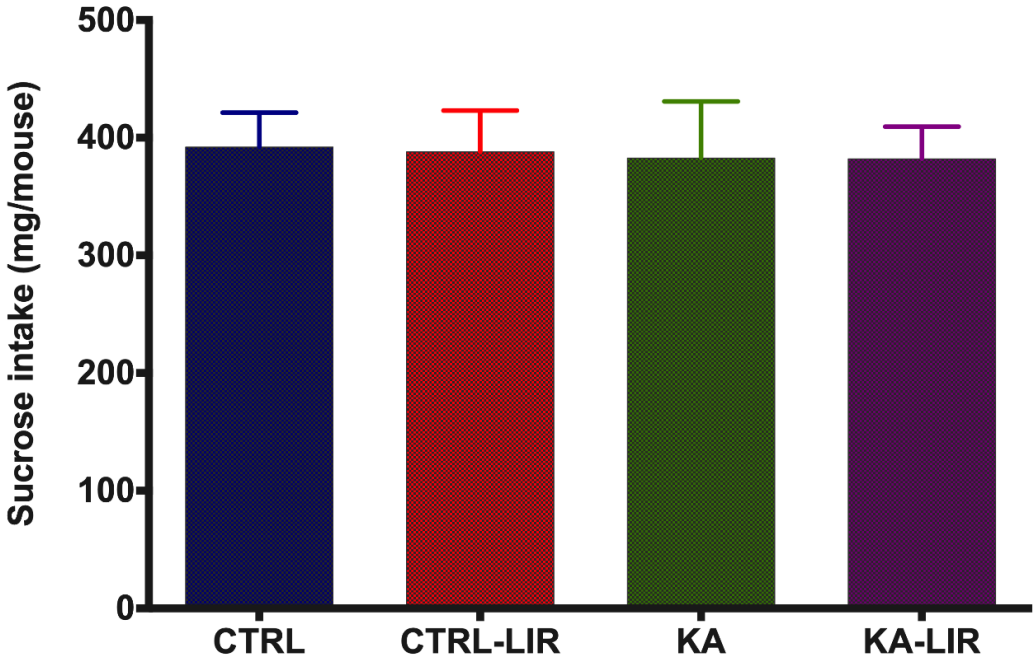

Fig. S1 Consumption of a 2% sucrose solution in 90 min expressed in milligrams (mg)/mouse. CTRL= Control mice; CTRL-LIR = Control LIR-treated mice; KA= kainate-treated mice; KA-LIR= KA-Liraglutide-treated mice.