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ROLE OF NITRIC OXIDE/cGMP PATHWAY IN CANNABINOID MODULATION OF PAROXYSMAL PHENOMENA

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INDEX

ABBREVIATIONS

2AG, 2-arachidonoylglycerol
7NI, 7-nitroindazole
AB, angular bundle
AC, associational commissural pathway
ACEA, 2'-chloroethylamide
AD, after discharge
AEA, anandamide
ANOVA, analysis of variance
ARG, arginine
BG, basal ganglia
CA1, *cornus ammonis* 1
CA3, *cornus ammonis* 3
cAMP/PKA, cyclic adenosine monophosphate/protein kinase A
CAP, capsaicin
CB, cannabinoids
CB_1R, cannabinoid receptor type 1
CB_2R, cannabinoid receptor type 2
CCK, cholecystokinin
cGMP, cyclic guanosine monophosphate
CNG, cyclic nucleotide-gated
CNS, central nervous system
CPZ, capsazepine
CTR, control
D%, % difference
DG, dentate gyrus
DSE, depolarization-induced suppression of excitation
DSI, depolarization-induced suppression of inhibition
eCB, endocannabinoids
GLU, glutamate
GP, globus pallidus
KA, kainic acid
LEC, lateral entorhinal cortex
LTD, long-term depression
LTP, long-term potentiation
mAChr, muscarinic acetylcholine receptor
MDA, maximal dentate gyrus activation
MEC, medial entorhinal cortex
MF, mossy fibers
NO, nitric oxide
NOS, NO synthase
eNOS, endothelial NOS
iNOS, inducible NOS
nNOS, neuronal NOS
NT, neurotransmitter
ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one
PKG, cGMP-dependent protein kinase
PLC, phospholipase C
PLD, phospholipase D
PP, perforant path
Sb, subiculum
SC, Schaffer collateral
SE, *status epilepticu*s
sGC, soluble guanylyl cyclase
STN, subthalamic nucleus
THC, tetrahydrocannabinol
TLE, temporal lobe epilepsy
TRPV1, transient receptor potential vanilloid type 1
WIN, (R)-(+)WIN 55,212-2.

INTRODUCTION

Temporal Lobe Epilepsy: focus on bioelectric substrates of hyperexcitability

Epilepsy is broadly recognized as a multi-factorial neurologic disorder, defined by a state of recurrent, spontaneous seizures (Stafstrom and Carmant, 2015). In patients, epileptic seizures are basically classified into generalized and focal (Scheffer et al., 2016). Generalized seizures engage the entire cortex, hence patients suffer from a complete loss of consciousness. Focal seizures originate from a localized brain region, thus retaining consciousness, unless they spread to neighbouring regions via local cortical connections, or to trans-hemispheric areas via long-association pathways such as the *corpus callosum*, in that case they secondarily generalize.

It is common thought that animal models of epilepsy are essential for better understanding the basic mechanisms of processes leading to epilepsy and therefore improving pharmacotherapy (Löscher, 2011). In acute models, epileptic seizures are provoked by electrical or chemical stimulation in naïve, non-epileptic animals, while the term 'chronic' refers to models that use animals that have been chronically made epileptic as above-described or animals with genetically-induced epilepsy (Fig. 1). Animal models of temporal lobe epilepsy (TLE) serve to expand knowledge on the most frequent type of focal seizure in adulthood (Wieser, 2004; Hauser et al., 1996) and share with human epilepsy many fundamental features. Remarkably, they are characterized by: *a)* localization of seizure *foci* within the limbic system, especially in the hippocampus and entorhinal cortex (Bartolomei et al., 2005); *b)* high incidence of hippocampal sclerosis (Mathern et al., 1997) and *c)* several molecular changes, including

alterations of genes and receptors (Bednarczyk et al., 2016; Karlócai et al., 2011; Ben-Ari and Dudek, 2010).

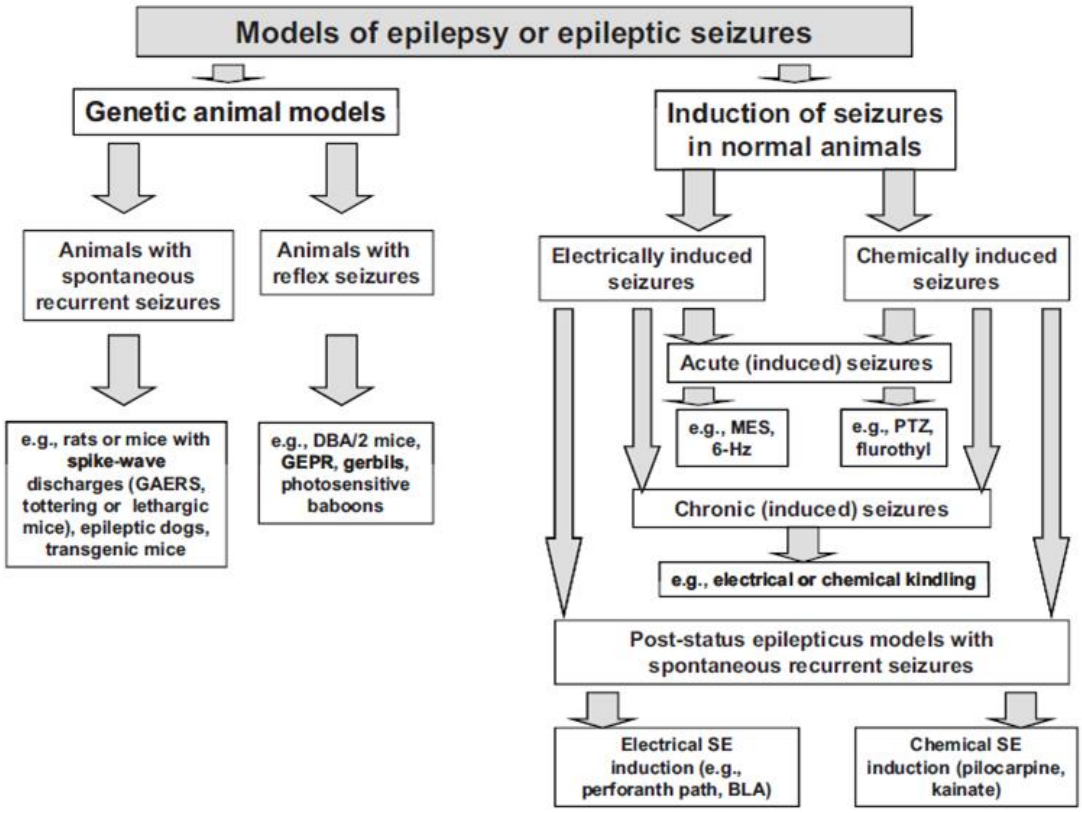

Fig. 1. Different epileptogenic mechanisms of seizures in animal models (Löscher, 2011).

Particularly, brain structures typically involved in experimental temporal lobe epilepsy and subjected to neuronal alteration are *cornus ammonis* 1 and 3 (CA1 and CA3) and the dentate hilus of the hippocampus, but the damage can also extend to extra-hippocampal regions, such as the entorhinal cortex or the amygdala (Ben-Ari and Dudek, 2010; Ben-Ari et al., 2008; 1980; see in fig. 2 a schematic representation of the hippocampal network). Similarly to mesial temporal sclerosis in human temporal lobe epilepsy, brain damage can either be quite limited (i.e. only a few hundred neurons loss) or wider, but patterns are variable across structures and animal models (Ben-Ari and Dudek, 2010).

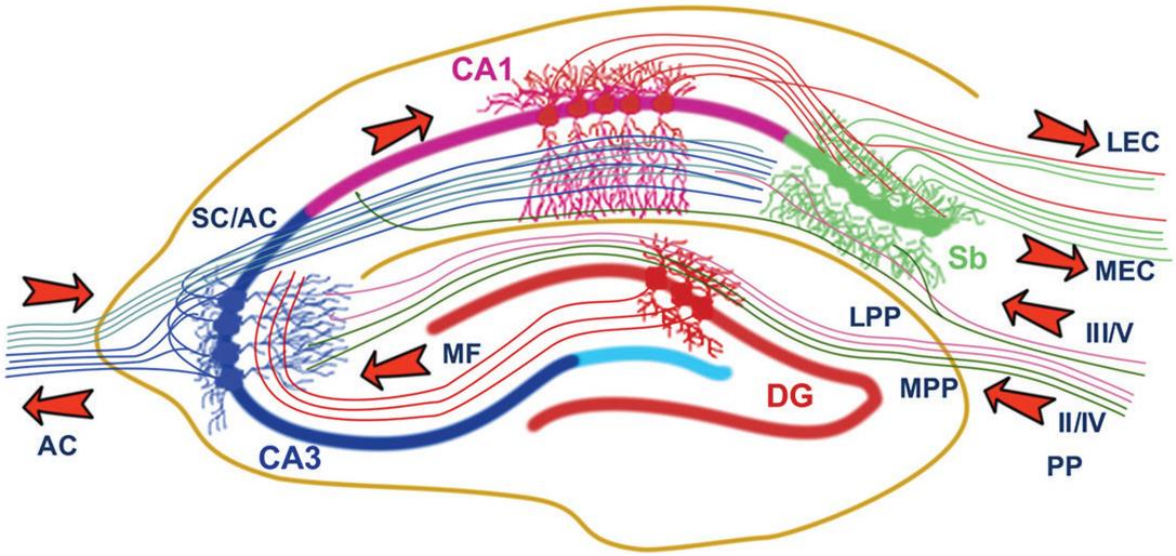

Fig. 2. Hippocampal network. The hippocampus receives inputs from the entorhinal cortex, that forms connections with the dentate gyrus (DG) and CA3 pyramidal neurons via the Perforant Path (PP). The axons of the PP arise principally in layers II and III of the entorhinal cortex and can be segregated into lateral and medial pathways (LPP and MPP, respectively), depending on whether the fibers arise from the lateral or medial entorhinal cortex (LEC and MEC, respectively). CA3 neurons receive input from the DG via the mossy fibers (MF). CA3 neurons provide axons to ipsilateral CA1 pyramidal neurons via the Schaffer Collateral Pathway (SC) and to contralateral CA1 neurons via the Associational Commissural Pathway (AC). Moreover, CA1 neurons receive input directly from the PP and send axons to the subiculum (Sb) and in turn send output back to the entorhinal cortex. (as in Sharma et al., 2007).

From a bioelectric point of view, dynamic changes occurring during epileptic seizures are rooted on altered excitability phenomena driving seizure initiation and propagation (Morelli et al. 2014; Bromfield et al., 2006). The initiation of seizures is defined by the concurrent presence of high-frequency bursts of action potentials and hyper-synchronized neuronal population. At single-neuron level, the burst of action potentials results from the "paroxysmal depolarizing shift" which consists of sustained, plateau-like neuronal depolarization that rapidly repolarizes and is followed by hyperpolarization. This prolonged depolarization of neuronal membranes is due to influx of extracellular Ca^{++}, leading to the voltage-channel mediated Na^{+} influx, that generates repetitive action potentials, whereas the subsequent

hyperpolarization is mediated by GABA receptor-dependent Cl^- influx, or by K^+ efflux. Bursting activity does not normally propagate during functional hyperpolarization that is supported by surrounding inhibition. Nonetheless, paroxysmal activation can *a)* increase extracellular K^+, attenuating hyperpolarizing outward K^+ currents, *b)* accumulate pre-synaptic Ca^{++}, enhancing neurotransmitter release, and *c)* activate NMDA subtype of glutamate excitatory receptor which sustains Ca^{++} influx and repetitive neuronal discharge. In this case, spontaneous rapid seizure termination fails and leads to the condition of *status epilepticus* (SE; Bromfield et al., 2006).

Network mechanisms are of noticeable importance for the regulation of hippocampal excitability, reflecting population activity of pyramidal cells and interneurons (Colgin, 2016; Gelinas et al., 2016). Epileptogenic regions are thought to reverberate the pathologically altered synchronization of neuronal assemblies (Foffani et al., 2007; Bragin et al., 2002; 2000). Indeed, epileptic seizures induce a local reorganization of inhibitory and excitatory circuits that modify population activity and brain oscillations (Foffani et al., 2007; Siddiqui and Joseph, 2005; Nadler et al., 1980), similarly to synaptic reorganization in humans with temporal lobe epilepsy (Wieser, 2004). The prevalent idea is that the mossy fibers of the dentate granule cells sprout and form novel excitatory synapses on neurons that they do not normally innervate (Ben-Ari and Dudek, 2010; Dudek and Sutula, 2007; Represa et al., 1987). This increased recurrent excitation in combination with the loss of vulnerable interneurons, in the dentate gyrus (DG) and CA1 as well, is a *fil rouge* along many temporal lobe epilepsy models, for example kindling and repetitive electric stimulation of the perforant path (Bengzon et al., 1997; Sloviter, 1983). Synaptic activity is therefore influenced by network phenomena that are driven and enhanced by aberrant glutamatergic firing and also by pre- and post-synaptic alterations in the GABAergic control (Siddiqui and Joseph, 2005; Nadler et al., 1980). In detail, the main glutamate (GLU) receptors involved in

epileptogenesis are: NMDA, especially NR1 subunit, upregulated in epileptic tissue in the hippocampus (O'Dell et al., 2012; Sandoval et al., 2011; de Moura et al., 2010) and kainic acid (KA) receptors, particularly GLU-K1 and GLU-K2 subunits (Ben-Ari et al., 2008; Epsztein et al., 2005). Though, only KA receptors operate the new aberrant synapses, whereas naïve ones rely on AMPA receptors (Ben-Ari et al., 2008; Epsztein et al., 2005). Loss of inhibitory control involves: qualitative and quantitative changes in $GABA_A$ receptor subunits (Fritschy et al., 1999); modulation by other neurotransmitters (Chamberlain et al., 2012; Oliveira et al., 2010) and phenotypic changes of receptor subtypes from hyperpolarizing to depolarizing activity (Galanopoulou, 2007).

In the light of this evidence, exploring the possible synaptic targets underlying pathophysiological alterations of neural transmission could promote advancement in the knowledge of hyperexcitability phenomena. Indeed, the modulation of neuronal transmission in the central nervous system (CNS) deserves great interest in orienting the development of new anticonvulsants towards targets that can influence bioelectric balance in neuronal processes. In this view, several elements have attracted attention so far. Among these, endocannabinoid system stands out for its variegated modulatory activity, exerted on classic receptor-mediated mechanism or on further molecular players such as nitric oxide.

Cannabinoid control of neuronal activity and epileptic seizures

Endocannabinoid (eCB) system contributes to synaptic function by various mechanisms and is involved in physiological processes such as memory formation, food intake, pain sensation and rewarded behaviour. Within the brain, CB_1R (cannabinoid receptor type 1) can be found in cortical and subcortical regions that are involved in superior functions, such as the basal ganglia, substantia nigra, hippocampus, cerebellum and brain stem. Classically, endocannabinoids modulate neurotransmitter signalling via a retrograde, feedback

mechanism. They are synthesized on-demand in the post-synaptic terminal, after elevation of Ca^{2+} levels, by Ca-dependent synthesizing enzymes. Then, eCBs are released in the synaptic cleft and target pre-synaptic CB_1R coupled to the Gi/o proteins (Howlett et al., 2002; Wilson and Nicoll, 2001). At the cortical level, CB_1R control neuronal activity by affecting both excitatory and inhibitory neurotransmission. In the hippocampus, the on-demand production of endocannabinoids from over-activated post-synaptic cells inhibits neurotransmitter release (Marsicano et al., 2003) specifically from two neuronal populations of CA1 area: cholecystokinin (CCK)-positive GABAergic interneurons and excitatory glutamatergic terminals onto pyramidal cells (Katona et al., 2006; Kawamura et al., 2006, Wilson and Nicoll, 2001). In accordance with this, CB_1R receptors can modulate stimulus-evoked inhibitory or excitatory post-synaptic potentials (Castillo et al., 2012; Ohno-Shosaku et al., 2001;Wilson and Nicoll, 2001; Wilson et al., 2001). In detail, pre-synaptic CB_1R can regulate short-term plasticity mechanisms, in which CB_1Rs are activated for a few seconds; then, the $\beta\gamma$ subunits of G-proteins inhibit pre-synaptic Ca^{2+} influx in the cytosol through voltage-gated Ca^{2+} channels (Brown et al., 2003; Kreitzer and Regehr, 2001; Wilson et al., 2001; fig. 3). Whereas, eCB-mediated long-term plasticity requires inhibition of adenylyl cyclase and downregulation of the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway (Castillo et al., 2012; Chevaleyre et al., 2006; fig. 3). In the context of retrograde eCB function, CB_2R (cannabinoid receptors type 2) distribution and function have been widely recognized in the immune system so far. Though, growing evidence support that CB_2R participates in a variety of brain processes (Den Boon et al., 2012; Morgan et al., 2009; Cabral et al., 2008; Fernández-Ruiz et al., 2007; Van Sickle et al., 2005). In particular, it is reported that CB_2R mediate an activity-induced self-inhibition in medial prefrontal cortex, decreasing neuronal firing (den Boon et al., 2012).

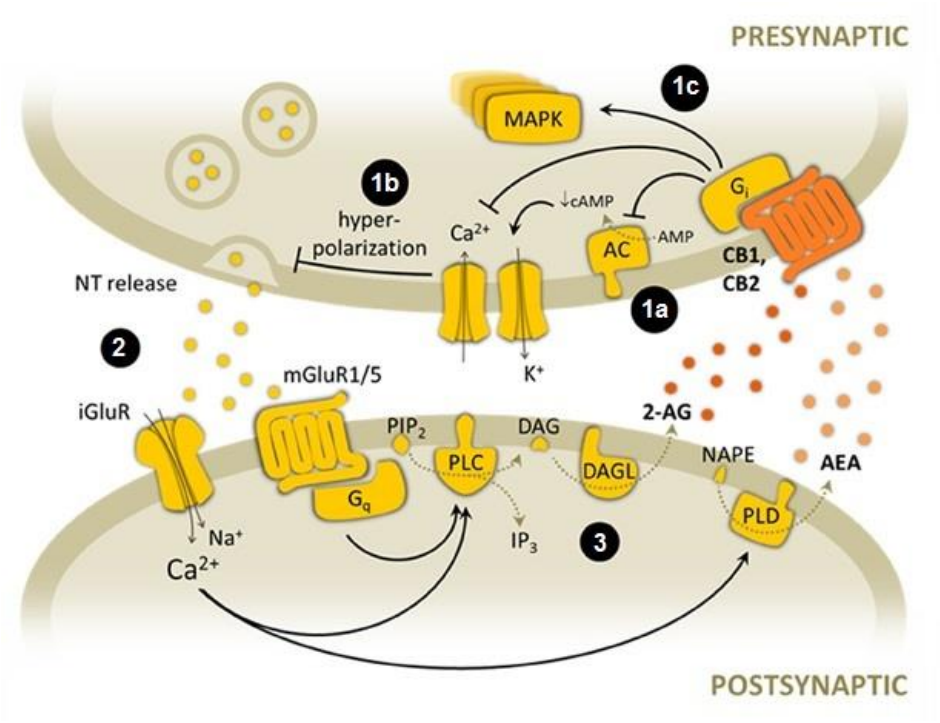

Fig. 3. Classical retrograde signalling mechanism of endocannabinoid system. Main downstream targets of CB receptors activation and subsequent Gi-protein stimulation are: (*1a*) inhibition of adenylyl cyclase (AC) activity, (*1b*) membrane hyperpolarization after modulation of K^+ and Ca^{2+} channels, (*1c***)** activation of protein kinase cascades such as MAPK pathway. These actions result in the inhibition of neurotransmitters (NT) release. (*2*) Usually, when glutamate is released from pre-synaptic terminals stimulates both ionotropic and metabotropic glutamate receptors, leading to post-synaptic depolarization through Ca^{2+} entrance and Gq-protein activation. (*3*) High Ca^{2+} concentration stimulates the synthesis of endocannabinoid (2-arachidonoylglycerol, 2AG, and anandamide, AEA) through phospholipase C and D (PLC and PLD). 2-AG synthesis is also mediated by Gq-protein activation. (*3*) Endocannabinoids are released to the synaptic cleft and activate CB_1 and CB_2 pre-synaptic receptors. (figure modified from Flores et al., 2013**).**

In addition, eCBs signal is thought to act in a non-retrograde manner recruiting the transient receptor potential vanilloid type 1 (TRPV1) and also CB_1Rs located on the post-synaptic side (Castillo et al., 2012). TRPV1 are non-selective cation channels, that integrate peripheral noxious stimuli activated by natural vanilloids (capsaicin and resiniferatoxin), heat and acids. TRPV1 channels, when gated, induce a complex cascade of events, including the release of pro-inflammatory mediators and neurotoxicity (Caterina et al., 1997). In particular, TRPV1

activation augments the membrane permeability to Na^+ and Ca^{++}, thereby modulating neuronal excitability (Saffarzadeh et al., 2015; Menigoz and Boudes, 2011; Szallasi and Blumberg, 2007; Cristino et al., 2006). TRPV1 are also reported to influence pivotal processes of the hippocampus by modulating mechanisms of synaptic efficiency such as long-term potentiation (LTP) and long-term depression (LTD) (Fu et al., 2009; Gibson et al., 2008; Leite et al., 2005).

Moreover, by presenting an additional intracellular binding site for anandamide (AEA, the major endocannabinoid agonist), TRPV1 has been considered as a possible "ionotropic receptor counterpart" for CB_1R and CB_2R, since many of pharmacological effects of AEA can be abolished by TRPV1 antagonism, knockout and desensitization (Ligresti et al., 2016). AEA and capsaicin share chemical and pharmacodynamic similarities as full agonists of TRPV1 (Szallasi and Blumberg, 2007), whereas other cannabinoids, such as (R)-(+)WIN 55,212-2 (hereafter, WIN) and 2-arachidonoylglycerol (2-AG), cannot be considered as TRPV1 classical agonists and their interference with this receptor is still to be elucidated (Di Marzo and De Petrocellis, 2012).

Considering eCB activity in regulating physiological neuroexcitability, it does not surprise that cannabinoids (CB) have caught attention as potential endogenous antiepileptics in the brain over the last decades (Hofmann and Franzier, 2013; Monory et al., 2006). Endogenous and exogenous cannabinoid agonists have demonstrated CBR-mediated anti-seizure effects after systemic administration in both epileptic animals and *in vitro* models (Blair et al., 2006; Wallace et al., 2003; 2001); whereas CB_1R antagonists prompt epileptic activity (Deshpande et al., 2007, Hofman and Franzier, 2013). In humans, rimonabant (CB_1R antagonist) triggered paroxysmal events in epileptic patients (Braakman et al., 2009) and a down-regulation of CB_1R was encountered in human epileptic tissue (Ludányi et al., 2008). In agreement, our laboratory previously yielded evidence that WIN, a CB non-selective agonist, exerts

antiepileptic effects in an *in vivo* model of hippocampal epilepsy; moreover, WIN was significantly, but incompletely, antagonized by CB_1R antagonist AM251 (Rizzo et al., 2009). The finding of AM251 ineffectiveness when administered alone and its partial antagonism on WIN-dependent activity conveyed the idea of the functional involvement of further mechanisms underpinning CB antiepileptic effects. In this regard, the action of eCBs, such as AEA, on TRPV1 was also found to influence epileptic events and to be challenged by CB_1R antagonist as well as by capsazepine, the TRPV1 antagonist (Di Marzo and De Petrocellis, 2012; Manna and Umathe, 2012; Bhaskaran and Smith, 2010; Chavez et al., 2010).

A predominant line of research supports that these protective effects by CB_1R agonists are specifically exerted onto excessive glutamatergic release from pyramidal cells (Monory et al., 2006; Marsicano et al., 2003). In this regard, cannabinoids are considered to impair normal synchronized network activity in the hippocampus, an action that was selectively ascribed to the suppression of pre-synaptic glutamate release from excitatory terminals (Maier et al., 2012). Therefore, cannabinoid influence on hippocampal network oscillations could play an important part in aberrant neuronal organization in hyperexcitability. At molecular level, CB_1R protection against excitotoxicity requires both hyperpolarization of neuronal membranes, mainly by increasing K^+ and decreasing Ca^{++} conductance that provide rapid protection, and the induction of intracellular cascades, for long-term adaptive changes. Nonetheless, psychoactive effects of cannabinoids have limited so far a comprehensive clinical assessment of treatment with phytocannabinoid agonists such as tetrahydrocannabinol (THC) and cannabidiol, though individual cases and limited studies tend to support their antiepileptic effects for development of symptomatic and prophylactic therapy (Soltesz et al., 2015, Cilio et al., 2014; Maa and Figi, 2014; Armstrong et al., 2009). Promising trials have been successfully carried out to evaluate cannabidiol therapeutic potential in untreatable paediatric epilepsies (Ligresti et al., 2016). Noteworthy, cannabidiol is thought to act on Ca^{2+}

homeostasis via different ways, one of which could be activation/desensitization of TRPV1 channels (Iannotti et al., 2014; Jones et al., 2012).

Besides the intracellular mechanisms above described, the eCB system has several, still unexplored points of interaction with other signalling systems, above all nitrergic transmission, that could be implicated in eCB protection from hyperexcitability phenomena.

Role of nitric oxide on neuronal excitability

Nitric oxide (NO) is a gaseous molecule synthesized from the oxidation of L-arginine by three different isoforms of NO synthase (NOS): the neuronal (nNOS) and endothelial (eNOS) constitutive isoforms are calcium activated; the inducible isoform (iNOS) is calcium-independent. NO targets the soluble guanylyl cyclase (sGC), a cyclic guanosine monophosphate (cGMP)-producing enzyme (Feil and Kleppisch, 2008), that acts downstream on cGMP-dependent protein kinases (PKG) or cyclic nucleotide-gated (CNG) ion channels, regulating intracellular Ca^{2+}. In the CNS, nitric oxide participates to neuronal function by regulating synaptic plasticity, axonal elongation and normal and pathological excitability (Ahern et al., 2002; Kiss and Vizi 2001; Prast and Philippu, 2001; Brenman and Bredt, 1997). Indeed, previous researches discovered the nitrergic modulation of neuronal activity in the rat basal ganglia (BG) area, comprising striatum, globus pallidus (GP) and subthalamic nucleus (STN) (Sardo et al., 2011; 2009; 2008; 2006). At molecular level, NO can impact on voltage-gated and ligand-gated channels either switching on the classical cGMP pathway or through protein modification (Garthwaite, 2008; Kiss, 2000). Furthermore, NO acts via Ca^{2+}-dependent and -independent processes affecting the release of neurotransmitters i.e. glutamate, GABA, acetylcholine, dopamine and noradrenaline (Ohkuma and Katsura, 2001; Arancio et al., 1995). Above all, NO interacts with the redox site of the glutamatergic NMDA receptor decreasing its response to agonists, especially during ‘‘over-activity’’ (Grima et al.,

2001; Quesada et al., 1996), and participates to metabotropic GLU receptors-dependent LTP (Anwyl, 2009). In the light of this, NO has been potentially considered to heavily influence the genesis and the spreading of the epileptiform hyperexcitability (Przegalinski et al., 1996), with several experimental observations that have revealed a general increase of NOS expression in various models of experimental epilepsy (Lumme et al., 2000; Hara et al., 1997). Though, no definitive conclusions have been released about a pro- or anti-convulsant role so far (Ferraro and Sardo, 2004; Borowicz et al., 2000; de Vasconcelos et al., 2000; Del-Bel et al., 1997).

Within the context of hyperexcitability, NO has caught attention since it likely represents a target of cannabinoid action and serves as a mediator for numerous CB effects (Bahremand et al., 2009; Jones et al., 2008; Kim et al., 2006 b), directly targeted by CB_1-activated pathway or by post-synaptic TRPV1 (Batista et al., 2015; Aguiar et al., 2014; Bredt and Snyder, 1989). In particular, the eCB system and cGMP signalling are reported to be functionally related in certain neuronal paradigms (Ghasemi et al., 2007; Howlett et al., 2004; Azad et al., 2001; Stefano et al., 1998). For instance, guanine nucleotides can inhibit CB agonists binding (Devane et al., 1988), whereas cannabinoid agonists can stimulate both the production of cGMP and the translocation of the NO-activated sGC (Jones et al., 2008). In rat microglial cells, a CB_1-mediated reduction of NO release, involving Ca-independent iNOS isoform, was demonstrated (Waksman et al., 1999). Particularly, in the hippocampus the distribution of these targets has been described in the pre-synaptic glutamatergic afferents (Burette et al., 2002). Anatomical evidence point to a co-localization of nNOS and NO-activated sGC in synapses supplied with CB_1 receptors (Burette et al., 2002; Azad et al., 2001), especially in the hippocampus (Makara et al., 2007). Furthermore, cannabinoids were reported to inhibit nNOS activity in brain processes, for instance blocking K^+-induced depolarization (Hillard et al., 1999) and reducing voltage-gated Ca^{2+} influx, therefore uncoupling membrane

depolarization from nNOS activation (Twitchell et al., 1997). Interestingly enough, it was found that neuronal nitric oxide could be engaged in the anticonvulsant properties of CB_1 receptor agonist arachidonyl-2'-chloroethylamide (ACEA), influencing seizure threshold in a behavioural model of epilepsy (Bahremand et al., 2009). Nonetheless, the linkage between cannabinoid and nitrergic systems in the modulation of hyperexcitability phenomena would deserve deeper investigations.

AIM

This research project aimed to deepen knowledge on the role of cannabinoid signalling in the context of epileptic hyperexcitability. The pharmacological manipulation of cannabinoid receptor pathway is a suitable approach for exploring the contribution of CB_1R and CB_2R in hyperexcitability phenomena, by applying specific exogenous CBR agonists and antagonists. Furthermore, the possible implication of nitric oxide in cannabinoid-activated pathways was investigated, taking into account its reported function as a neuronal mediator. This was put into practice by evaluating cannabinoid effects in presence of several NO-active drugs that could reduce or increase NO activity, modifying its production and blocking its downstream target, the sGC. Lastly, TRPV1 role on CB antiepileptic effects was assessed by a pharmacological characterization of capsaicin and capsazepine alone and in combination with a CB agonist, within the framework of paroxysmal phenomena induced in hippocampal models of epilepsy. The ultimate goal was to propose putative points of interaction between the systems involved in the neuromodulation of hyperexcitability, focusing on the linkage among CB receptors, TRPV1 and nitric oxide.

Two models of acute epileptiform activity were used, the maximal dentate activation (MDA) and the acute pilocarpine-induced paradigm, respectively characterized by electrically and chemically-evoked seizures (Löscher, 2011; Raza et al., 2001; Mello et al., 1993). In the MDA, free of chemical interferences, an excitatory re-entrant loop in the limbic system is activated by electric stimulation of the angular bundle (AB) (Stringer and Lothman, 1992). This model is defined by the presence of electrically-induced hippocampal bursts of large amplitude population spikes, associated with a secondary rise in the extracellular K^+ and a

negative shift of the DC potential (Stringer and Lothman, 1992). The synchronous burst discharge of dentate cells, after hyper-activation of a hippocampal–parahippocampal circuitry, constitutes a bio-electric marker of acute TLE in the hippocampus that is extensively studied as a crucial focus of hyperexcitability processes (Kandel et al., 2014; Stringer and Lothman, 1989). In the behavioural model of TLE, pilocarpine elicits cholinergic-induced epileptogenesis by activating muscarinic receptors in the hippocampus and causes spontaneous seizures with typical behavioural symptoms (Curia et al., 2008). The pilocarpine model has been widely employed since it reproduces the characteristics of acquired, refractory human epilepsy, such as anatomical damage, network reorganization and pharmacoresistance to conventional antiepileptic drugs (Curia et al., 2008; Hamilton et al., 1997; Mello et al., 1993).

Noteworthy, studies about the pathophysiology of epileptic phenomena may produce conflicting results, for instance due to different experimental protocols, animal species and pharmacological properties of drugs chosen (Löscher, 2011; Löscher et al., 1991). For this reason, when feasible, it was designed an integrated experimental approach, coupling electrophysiological and behavioural methods to evaluate the effects of perturbing cannabinoid neuromodulatory activity on the genesis and maintenance of epileptic activity in the hippocampus, in both anesthetized and freely-moving animals. Comparing pharmacological outcomes from these acute TLE models that have distinct epileptogenic triggers, allowed to place robust relationship between CB/TRPV1 system and nitric oxide signalling within the remit of hippocampal hyperexcitability. This research, investigating on several factors underpinning the neurobiological mechanisms of cannabinoid control of seizures, may promote the development of novel, non-conventional therapeutic perspectives.

MATERIALS AND METHODS

Animal experiments were conducted in strict accordance with the European Directive on animal experimentation (2010/63/EU) and the institutional guidelines, authorized by the Italian Ministry of Health. All efforts were made to minimize animal suffering and to reduce the number of animals used.

EXPERIMENTAL MODELS OF TLE

MDA model

Surgical procedures

Male Wistar rats (weight 260–300 g, 2–3 months-old) were anesthetized with urethane (1.2 g/kg intraperitoneally, i.p.). The trachea was cannulated and the skull exposed. The animals were positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and the body temperature was maintained at 37–38°C using a heating pad. Heart rate and pupil diameter were monitored during all the experimental session. A craniotomy was performed to expose a wide area of the right cerebral cortex, then the dura was removed. A stimulating depth electrode (coaxial bipolar stainless steel electrode: external diameter 0.5 mm; exposed tip 25–50 µm) was placed in the AB on the right side according to the stereotaxic coordinates of the Atlas of Paxinos and Watson (1986) (AB: 1 mm anterior to the interaural line; 3–5 mm dorsal to it and 4.4 mm lateral to the midline). A glass recording microelectrode (1–2 µm tip diameter, 1.8–2.2 MΩ electrode resistance), filled with 1% Fast Green in 2 M NaCl, was stereotaxically placed in the DG on the right side (DG: 6 mm anterior to the interaural line;

3.0 mm ventral to the cortical surface and 1.8 mm lateral to the midline). The animal was grounded through a subcutaneous Ag/AgCl wire in the scapular region.

Electrophysiological procedures

The protocol of MDA originally designed by Stringer and Lothman (1992) was previously modified by introducing: a fixed duration of the electrical stimulation, a related way to determine the threshold and the stimulation intensity, and a fixed spacing between stimulations (Ferraro and Sardo, 2009; Sardo et al., 2009; 2008; 2006).

Fixed duration (10 s) trains of 20-Hz stimuli were given through the AB stimulating electrode. Individual stimuli consisted of 0.3ms biphasic pulses; the stimulus intensity was initially below that necessary to elicit any response and it was increased by 100μA steps in the following stimulations until MDA occurred (threshold intensity). The stimulus train was administered every 2 minutes until a MDA appeared and then every 10 minutes for up to 3 h. MDA was recorded by the electrode placed in the DG and it was defined by a shift of the extracellular potential in DC-coupled recordings as well as by the presence of bursts of population spikes (Fig. 4). Once the MDA was elicited, the percentage of responses to AB stimulation was analysed to appraise the effect of pharmacological treatment; the eventual absence of MDA response per group (Fig. 7 c) was taken into consideration to evaluate the percentage of protection (% protection) against electrically induced epileptiform events on the basis of the following formula: 100 * (total number of no responses to stimulation per group/total number of stimulations per group). Furthermore, three parameters describing the electrophysiological features of MDA were monitored along each experimental session: time of onset (latency) as an indicator of the susceptibility of the DG to respond to stimulation; MDA and after discharge (AD) duration to quantify the extent of epileptic discharge (see fig. 4). In detail, onset is time from the beginning of AB stimulation to the midpoint of the DC

potential shift; MDA duration is from the midpoint of the DC potential shift to the point at which the evoked paroxysmal EEG events abruptly ceased and AD is from the end of AB stimulation to the end of the epileptiform activity.

The DG bioelectric activity was recorded through a low-level DC pre-amplifier (Grass 7B, West Warwick, RI, USA) and then processed by the SciWorks 5.0 package provided by DataWave Technologies (Longmont, CO, U.S.A.).

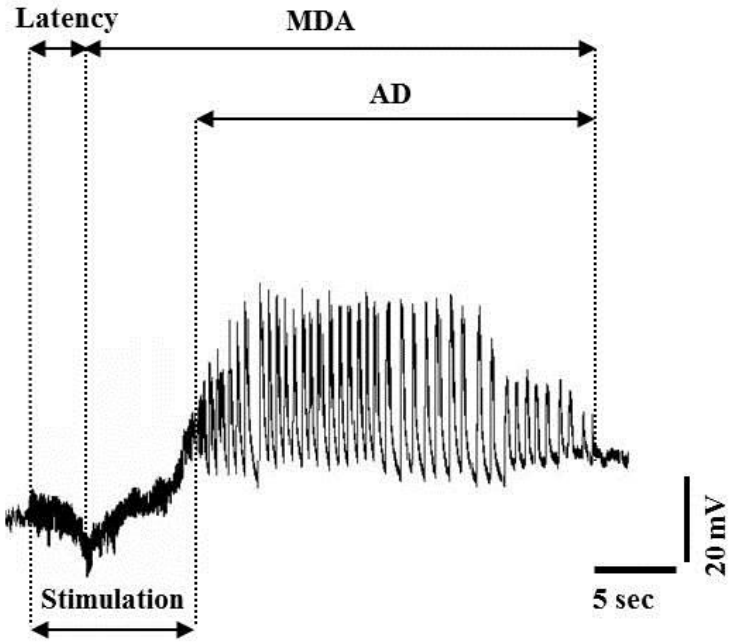

Fig. 4. Representative MDA trace. Measurements of time of onset (latency), duration of maximal dentate gyrus activation (MDA) and after discharge (AD) during and after a stimulus train (400 µA, 20 Hz) delivered for 10 s to the angular bundle (AB).

Histological verification

Recording and stimulating electrode positions were respectively marked through iontophoretic Fast Green ejection (50 µA for 10 min) and a small electrolytic lesion (20 mA for 10 s.), then they were histologically verified. On completion of each experiment, the

animals were anesthetized by an overdose of pentobarbital i.p. and whole-body perfused with normal saline, followed by 10% buffered formalin. The brains were removed, post-fixed in the same fixative overnight and then cryoprotected in 30% sucrose/PBS. Finally, brains were sliced in 30–50-µm serial coronal sections and stained by using Nissl-Cresyl Violet method (Sardo et al., 2008; fig. 5).

Statistical analysis

A Chi-square (χ^2) test was used to compare the % of responses to AB electrical stimulation following each drug treatment within the same experimental group and between treatments. For co-treated groups, the % of MDA responses was taken into consideration from the last drug administration.

For the studied parameters (the time of onset, MDA or AD durations), data from each animal were expressed as % difference (D%) versus the baseline value measured in the last MDA response preceding vehicle or drug injection (Fig. 6), considered as the reference value for within-group statistical comparisons. Then, in each group D% data were averaged per stimulation on the basis of time elapsed from the first stimulation following the treatment and then plotted as mean D% ± S.D. (see figure legends). For significant changes, maximum mean D% and the related mean absolute values are reported. The time course of MDA parameters was analysed within each group using a one-way analysis of variance (ANOVA) versus baseline values, with a following Bonferroni post-hoc test. Between-treatments comparisons were made by two-way ANOVA, since the occurrence of drug-induced suppression of paroxysmal response did not allow the use of a repeated measures ANOVA, and by Bonferroni post-hoc test. This analysis was employed to assess the effect of time of stimulus (as within-group factor), treatment (as between-groups factor) and their interaction

on MDA parameters. Differences were considered statistically significant when P was less than 0.05.

Pilocarpine model

Behavioural procedures

The acute pilocarpine model was used to induce seizures by well-established procedures (Cuellar-Herrera et al., 2010; Falenski et al., 2009; Mello et al., 1993). Briefly, adult male Wistar rats (weight 260–300 g) were administered with methyl-scopolamine (1 mg/kg, i.p.) 30 minutes before pilocarpine treatment to minimize peripheral, parasympathetic effects. Pilocarpine (350 mg/kg, i.p.) was then given to induce seizures, whose onset typically occurred within 20 to 40 min after pilocarpine injection. Behavioural seizures were monitored for 120 minutes by video recording, scored off-line every 10 minutes and confirmed by an experimenter blinded to the experimental treatment. A seizure severity score (0–5) was assigned according to a modified Racine scale, appropriate for the acute pilocarpine model (Jones et al., 2012), with regard to the animals' maximal behavioural response (as indicated in the table 1). The time course of these scores was used as a qualitative measurement of the severity of the convulsions the ''behavioural scoring''. On the whole, the following parameters were assessed during the observation: (1) behavioural scoring; (2) latency to the first seizure state; (3) percentage of animals presenting severe generalized seizures, i.e. tonic-clonic seizures; (4) mortality rate within 24 h, as assessed by Cuellar-Herrera et al. (2010).

Acute pilocarpine-induced temporal lobe seizures		
Seizure score	Behavioural expression	Righting reflex
0	No change in behaviour	Preserved
1	Mouth clonus	Preserved
2	Unilateral forelimb clonus	Preserved
3	Bilateral forelimb clonus	Preserved
4	Bilateral forelimb clonus with rearing and falling	Preserved
5	Tonic–clonic seizure	Lost

Tab. 1. Severity scoring scale for acute pilocarpine-induced seizures (Jones et al., 2012).

Statistical Analysis

Results of total behavioural changes produced by pilocarpine injection were compared using the non-parametric Kruskal–Wallis ANOVA, applied for the ordinal variables (total seizure severity scored with the Racine scale), followed by a post-hoc Dunn's test. The differences between behavioural scores per time point in control and treated groups were statistically analysed using the Fisher's exact test. Furthermore, latencies to first seizure state (in minutes) were analysed using a parametric one-way ANOVA for within- and between-treatments differences, followed by a Bonferroni post-hoc test for multiple comparisons. Lastly, the percentage of animals with severe generalized seizures and mortality rate were analysed using the χ^2 test. Data are presented as mean ± S.D. or percentages. Values were considered statistically significant when $P<0.05$.

EXPERIMENT 1: CB AND NO-ACTIVE DRUGS ASSESSMENT

MDA model

Drug treatment

All drugs were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA), with the exception of AM251, purchased from AbCam (Cambridge, UK), and of AM630 and ODQ (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) from Tocris Bioscience (Bristol, UK). All drugs were dissolved in the same vehicle for each animal (15% of DMSO in saline solution).

In experiment 1, several groups of rats were taken into consideration. Control and vehicle-treated groups were studied for a period of at least 250 minutes in order to verify possible lack of response or modifications of MDA parameters due to the repetitive stimulations or to the vehicle administration. Responses of vehicle-treated rats were not different in comparison with controls.

Cannabinoid drugs used in experiment 1 were as follows: WIN, a CBR non selective agonist; AM251, a CB_1R antagonist, and AM630, a CB_2R antagonist. Whereas, the NO-active substances applied were: 7-nitroindazole (hereafter 7NI), preferential nNOS inhibitor; arginine (hereafter, ARG), precursor of NO synthesis, and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (hereafter named ODQ), specific sGC antagonist.

Firstly, groups were administered with single treatments of cannabinoids at the following doses: WIN in a range from 1 to 21 mg/kg, i.p. and AM630 at 2 mg/kg (n=6 per group). Also, single administration of 7NI were injected at increasing doses from 20 to 50 mg/kg i.p. per group (n=10 each). Then, co-treatment experiments between drugs at effective doses were performed to assess putative interactions in the following groups: AM630-WIN, ODQ-WIN and ARG-WIN groups (n=6 per group, with ODQ injected at 10 mg/kg and ARG at 1 g/kg). Furthermore, a co-treatment was administered with subeffective doses of 7NI and WIN (20

and 5 mg/kg, i.p. respectively) and indicated as 7NI-WIN group (n=10 rats). Lastly, the injection of AM251 (1 mg/kg, i.p.) was performed before subeffective doses of 7NI and WIN and indicated as AM251-7NI–WIN group (n=8 rats) to evaluate CB_1 receptors influence on the interaction. All treatment dosages were based on previous studies and pilot experiments (García-Gutiérrez et al., 2012; Rizzo et al., 2009; Sardo et al., 2008; 2006; Wallace et al., 2001). Each pharmacological treatment was performed after five consecutive stable MDA responses (baseline period) and the subsequent observation period lasted up to 180 minutes after the injection of the last drug. In the co-treatment groups, due to different pharmacokinetic profiles of the drugs administered, an interval was interposed between administrations so as to allow coincident actions (all drugs 30 min from WIN injection and AM251 50 min before 7NI-WIN).

Pilocarpine model

Drug treatment

Behavioural procedures in experiment 1 comprised 10 rats per group. Control and vehicle-treated groups were studied for a period of 120 minutes in order to verify possible modifications of behavioural seizures due to vehicle administration. In each remaining group, prior to behavioural seizure protocol, animals received the same individual doses of 7NI and WIN, as in MDA model. Single pharmacological treatment with 7NI or WIN at different doses was performed 15 minute before scopolamine administration (Jones et al., 2012). Control tests to assess the effects of AM251 as an antagonist of WIN action (AM-WIN group) were performed administering AM251 (1 mg/kg, i.p.) 40 min before an effective dose of WIN (10 mg/kg). In the group indicated as 7NI-WIN, animals were co-treated with sub-effective doses of 7NI (20 mg/kg, i.p) and WIN (1 mg/kg, i.p.). WIN was injected 15 minute before

scopolamine at distinct times from 7NI on the basis of kinetics of action (7NI 20 min before WIN injection). As for AM251-7NI–WIN group, AM251 (1 mg/kg, i.p.) was added to 7NI–WIN administration protocol, with the AM251 injected 40 min before WIN. To exclude the effect of vehicle, co-treatment experiments were preliminary conducted administering the vehicles before WIN.

EXPERIMENT 2: TRPV1 ROLE IN CANNABINOID EFFECTS

MDA model

Drug treatment

All drugs were purchased as above, whereas capsaicin (CAP) and capsazepine (CPZ) were purchased from ABCam (Cambridge, UK). This experiment took into consideration groups of animals, comprising 6 rats each. In the single-treated groups the animals received CAP in a range from 1 to 10 mg/kg, i.p., respectively per group, and CPZ from 0.5 to 2 mg/kg, i.p. Then, co-treatments were performed in the following groups: a co-administration with CAP and WIN (both at 10 mg/kg, i.p), a co-administration with CPZ and WIN (2 mg/kg and 10 mg/kg, i.p, respectively) and a co-administration with CPZ and WIN (2 mg/kg and 5 mg/kg, i.p, respectively). Dosages chosen for WIN co-administration are described above (see Experiment 1), while TRPV1-active drugs were applied on the basis of previous findings (Jia et al., 2015). All drugs were dissolved in the same final vehicle volume for each animal (15% of DMSO in saline solution). In the co-treated groups, due to different pharmacokinetic profiles of the drugs administered, an interval was interposed between administrations so as to allow coincident actions. In detail, the CAP-WIN group was administered with CAP 30 min before WIN injection, and the CPZ-WIN groups with CPZ 30 min before receiving treatment with WIN at 10 or 5 mg/kg.

Lastly, pilot experiments were conducted administering 7NI, CAP and WIN (at 50, 10 and 10 mg/kg, respectively), and ARG, CAP and WIN (1g/kg for ARG and 10 mg/kg for the other two). In these 2 groups (7NI-CAP-WIN and ARG-CAP-WIN groups), 7NI or ARG were injected respectively 10 min before CAP and 40 min before WIN injection.

For all the experiments, each pharmacological treatment was performed only after five consecutive stable MDA responses (baseline period) and the subsequent observation period

lasted 180 minutes after the drug injection in the groups treated with single drugs or after the last drug administration for all co-treated groups.

RESULTS

EXPERIMENT 1

MDA model

Control and vehicle-treated groups

In untreated controls and vehicle-treated groups, repetitive AB stimulations always induced a MDA response whose parameters were not altered along the experimental observation period (Tab. 2, fig. 6 and 7 a).

Time of stimulus	CTR	WIN 10 mg/kg	WIN 5 mg/kg	WIN 1 mg/kg	AM630-WIN
10	100	100	100	100	83
20	100	33**	83	100	67
30	100	50*	83	100	0**
40	100	50*	67	100	16**
50	100	33**	100	100	16**
60	100	83	100	100	50*
70	100	83	100	90	67
80	100	67	100	100	83
90	100	67	100	100	83
100	100	67	100	100	50*
110	100	67	100	100	83
120	100	67	100	100	100

Tab. 2. Summary of the percentage (%) of MDA responses per group, considering the first 120 min of stimulation. The reported significances are derived from a χ^2 analysis. (*) $P < 0.05$. (**) $P < 0.01$ versus vehicle-treated controls (CTR).

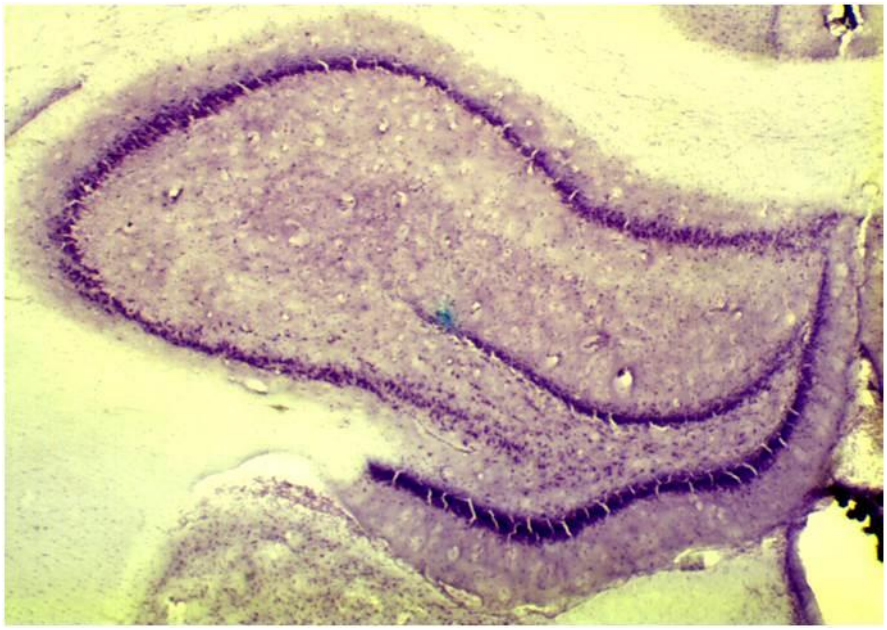

Fig. 5. Histological hippocampal section: 1.7mm (left) and 5.4mm (right) anterior to the interaural line (photomicrograph, 40x; Nissl-Cresyl Violet staining). The green dot shows a recording site in the dentate gyrus (DG) marked through iontophoretic Fast Green injection.

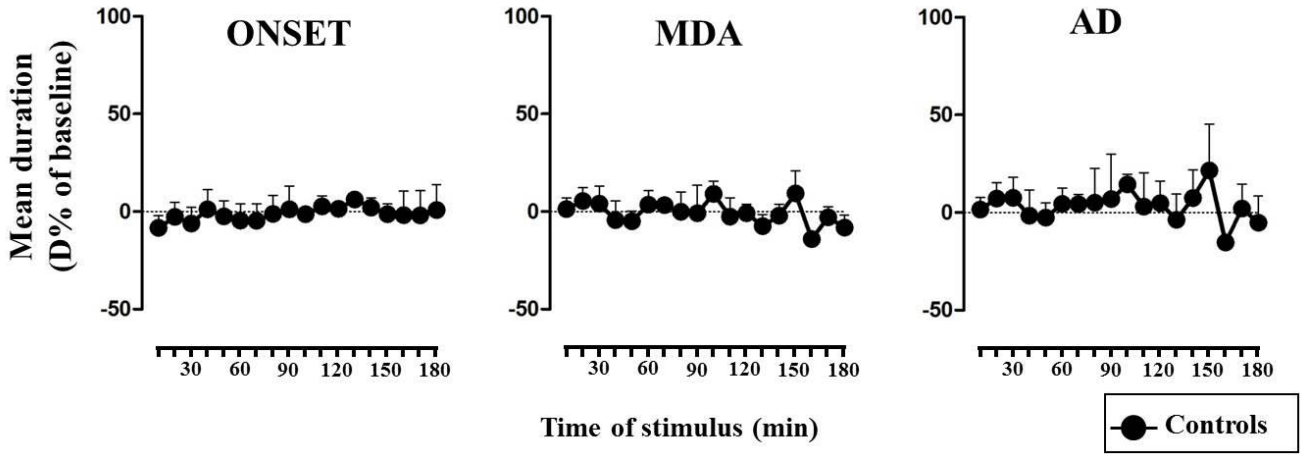

Fig. 6. Time course of MDA parameters in vehicle-treated controls. Each value represents the mean D% of baseline ±S.D.

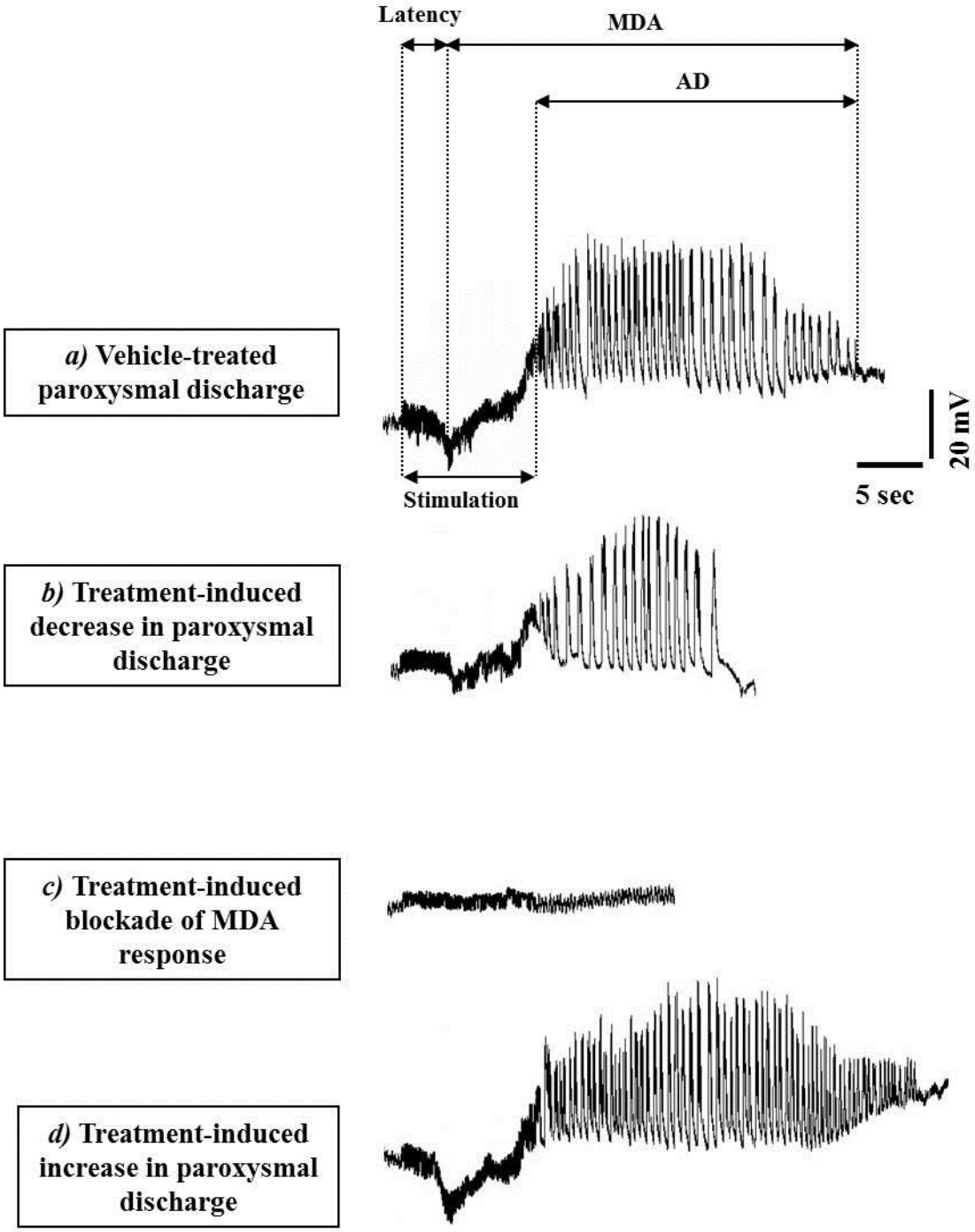

Fig. 7. Representative MDA responses exhibiting: *a)* vehicle-treated paroxysmal discharge; *b)* treatment-induced decrease in paroxysmal discharge; *c)* treatment-induced blockade of paroxysmal discharge; *d)* treatment-induced increase in paroxysmal discharge. Example traces are taken from the same animal.

Effect of single doses of WIN and of AM630 on % protection and MDA parameters

A within-treatment analysis on MDA responses, after WIN administration at different dosages, highlighted a clear efficacy (% protection = 27, 77%) exerted by WIN at 10 mg/kg (Fig. 8 a). The amount of non-responses was significant up to 50th min with a maximal effect at 20th and 50th min when only 33.33% of animals responded to AB stimulation (χ^2= 6.00, DF = 1, P = 0.0014; tab. 2 and represented in fig. 7 c). The whole period of protection last up to 140th min. On the other hand, WIN 5 mg/kg showed a non-significant protection in a fewer number of stimulations (20–40th min; tab. 2). Then, analyses were conducted on WIN effect on MDA parameters. In the group treated with WIN at 10 mg/kg, a within-treatment ANOVA revealed an increase in the time of onset ($F_{(18,65)}$ = 2.07, P = 0.017; fig. 8 b; as represented in fig. 7 b). Post-hoc analysis showed a significant effect from 20th to 130th min, with a maximum effect at 60th min (D% = +61.78 ± 17.56). Moreover, a reduction of MDA duration was evident ($F_{(18,65)}$ = 3.79, P < 0.0001), starting from 20th to 110th min with a maximum effect at 60th min (D% = −69.72 ± 32.23). Similarly, WIN 10 mg/kg induced a reduction in the AD duration ($F_{(18,65)}$ = 2.88, P < 0.0001), from 20th to 110th min, with a maximum effect at 60th min (D% = −71.68 ± 32.88). As for the treatment with WIN at 5 and 1 mg/kg, a within-treatment ANOVA did not outline any statistical differences. Between-treatments comparisons of WIN 10 mg/kg versus both WIN at 5 mg/kg and controls confirmed that significant differences were found for all parameters. Indeed, WIN at 10 mg/kg showed significant main effects versus WIN at 5 mg/kg of stimulus time (Onset: $F_{(17,180)}$ = 7.721, P<0.0001; MDA: $F_{(17,180)}$ = 4.193, P<0.0001 and AD: $F_{(17,180)}$ = 3.742, P<0.0001), treatment (Onset: $F_{(17,180)}$ = 475.7, P< 0.0001; MDA: $F_{(17,180)}$ = 88.81, P<0.0001 and AD: $F_{(17,180)}$ = 34.12, P<0.0001) and interaction (Onset: $F_{(17,180)}$= 4.205, P<0.0001; MDA: $F_{(17,180)}$= 2.239, P=0.0047 and AD: $F_{(17,180)}$ =1.747, P=0.0038). Between-treatments post-hoc results showed

an increase in the onset time in WIN at 10 mg/kg and subsequent reduction in MDA and AD durations for P<0.05 (as indicated in fig. 8 b).

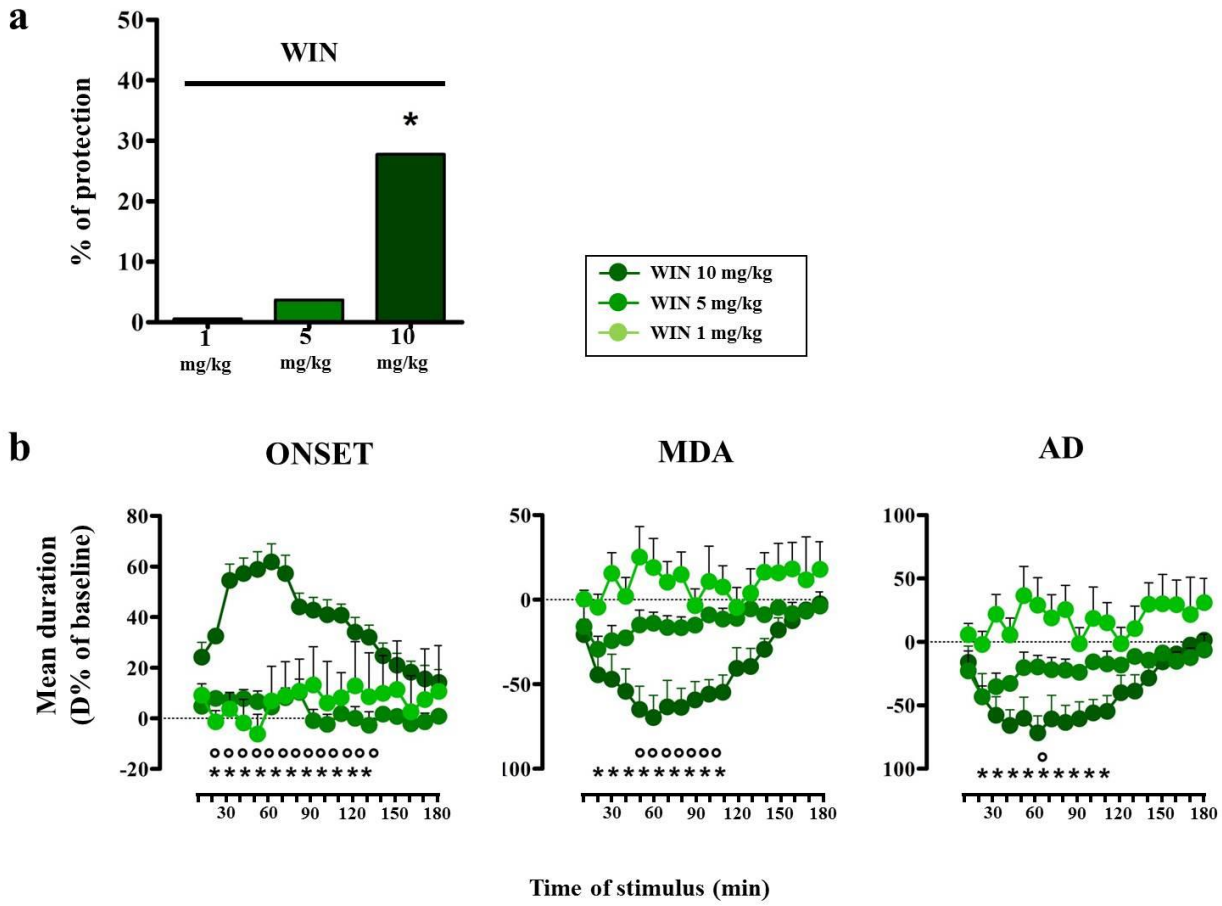

Fig. 8. *(a)* Effects of WIN at 10, 5 and 1 mg/kg on the % of MDA protection. (*) for the experimental groups that presented significant differences at least in one stimulation time point, versus controls (for P<0.05). *(b)* Time course of MDA parameters during the 18 progressive stimuli. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (● WIN 10 mg/kg, ● WIN 5 mg/kg, ● WIN 1 mg/kg). Within-treatment statistically significant D% of WIN at 10 mg/kg is indicated as (*) for P<0.05 vs baseline values. Between-treatments significance of WIN at 10 mg/kg was indicated as (°) for P<0.05 versus WIN at 5 mg/kg.

The co-treatment of AM630 and WIN at maximal dose enhanced WIN-induced reduction in the % of responses to AB stimulation, as described henceforth. In particular, a within-treatment analysis on AM630-WIN group displayed significant changes on the percentage of responses to AB stimulation. Indeed, data showed a marked decrease from 30th to 60th min

and from 100th to 110th min, with a maximal effect at 30th min when no animals exhibited any MDA response (χ^2= 12.000, DF=1, P=0.0005). A further analysis on the % of responses in AM630-WIN group showed significant differences with respect to WIN alone: in fact, the co-treatment continuously reduced the % of responses from 30th to 70th min, with a significant decrease at 3rd stimulus (maximal reduction observed: 50%, χ^2=4.000, DF=1, P=0.0455), with respect to WIN alone (Tab .2). The assessment of AM630 alone proved to be ineffective on MDA responses since AB stimulations were always followed by DG activation in the observation period. The pre-treatment with AM630 in WIN-treated animals enhanced WIN effect on MDA parameters. In particular, a within-treatment analysis revealed that AM630-WIN co-treatment increased the mean onset from 60th to 110th min, with a maximum effect of +86.05 ± 10.22% at 70th min ($F_{(15,55)}$= 10.754, P= 0.0003). Moreover, a reduction of MDA duration was evident from 60th to 120th min, with a maximum effect of -85.49 ± 3.92% at 70th min ($F_{(16,51)}$= 6.212, P= 0.0001). Similarly, the co-treatment induced a reduction in the AD duration from 60th to 120th min, with a maximum effect of +87.04 ± 3.20% at 70th min ($F_{(15,55)}$= 5.994, P= 0.0003). Lastly, a statistical comparison between the effects of AM630-WIN versus WIN alone showed a significant increase of the time of onset from 60th to 120th min in the co-treated group, with the maximal effect at 110th of +80.56 (from –8.76% ± 15.2 to +71.8% ± 13.9; $F_{(1,6)}$= 59.236 and P= 0.0003;). The same analysis for MDA and AD durations did not reveal significant differences (Fig. 9).

The possible variations of MDA parameters was also evaluated in the group treated with AM630 alone. It was found that the treatment did not significantly modify MDA parameters, if compared to baseline period. Also, no statistical significance resulted from a between-treatments analysis comparing MDA parameters in AM630 versus vehicle-treated and control group. Furthermore, a statistical analysis was performed to compare the effects of AM630 versus WIN administration at the highest dose used on MDA parameters. Significant

differences did not emerge in the time of onset whereas a significant reduction of the mean MDA duration was found in WIN group from 40th to 100th min, with a maximum effect at 60th min of -54.79 (from -15.6 ± 23.82% to -70.39 ± 13.70%; $F_{(1,9)}$= 10.033 and P= 0.0114;), compared to AM630. Lastly, it was shown a significant decrease of AD duration in WIN group from 40th to 100th min, with a maximum effect at 90th min of -85.13 (from +17.65 ± 42.51% to -67.48 ± 10.86%; $F_{(16,51)}$= 13.662 and P= 0.007), compared to AM630 (Fig. 9).

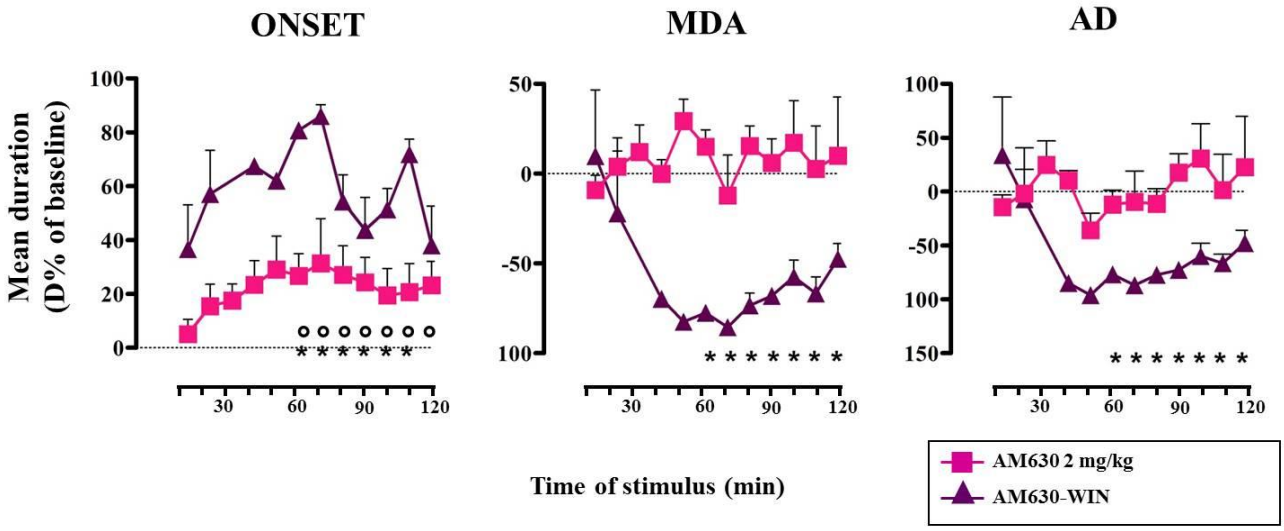

Fig. 9. Effects of AM630 at 2 mg/kg and AM630-WIN on the time course of MDA parameters during the 12 progressive stimuli. Each value represents the mean of D% of each treatment (■ AM630, ▲ AM630-WIN) per stimulus versus baseline values. At 30th min in AM630-WIN group no animal responded to stimulation, therefore this value is not reported in figure. Within-treatment statistically significant D% of AM630-WIN is indicated for P < 0.05 (*) vs baseline values. Between-treatments significance of AM630-WIN is indicated for P < 0.05 (°) vs WIN alone.

Effect of single doses of 7NI on % protection and MDA parameters

Within-treatment analysis on the group treated with 7NI 50 mg/kg displayed significant changes in the percentage of responses to AB stimulation. Indeed, data showed a marked decrease from 40th to 150th min, with a maximal effect at 100th and 120th min when 40% of animals were protected from seizures induced in DG by AB stimulation (χ^2=5.0, DF=1,

P=0.025). In contrast, the treatment with 7NI 35 mg/kg did not show any statistically significant percentage of protection, though MDA was not elicited in the 10% of animals only at 20th min, and in some stimulations after 110th min. Lastly, the treatment with 7NI 20 mg/kg resulted clearly inefficacious since all animals in this group always responded to all stimulations (Tab. 3 and fig. 10 a).

Time of stimulus	**7NI** 50 mg/kg	**7NI** 35 mg/kg	**7NI** 20 mg/kg	**7NI-WIN**	**AM251-7NI-WIN**	**ARG-WIN**	**ODQ-WIN**
10	100	100	100	50**	100	100	33*
20	100	90	100	20***	100	83	16**
30	100	100	100	20***	100	66	16**
40	90	100	100	20***	100	66	16**
50	70	100	100	40**	90	66	16**
60	70	100	100	60*	90	83	33*
70	80	100	100	60*	100	100	16**
80	70	100	100	80	100	100	16**
90	70	100	100	70	100	83	33*
100	60*	100	100	100	100	83	16**
110	70	90	100	80	100	83	33*
120	60*	90	100	100	100	100	50*

Tab. 3. Summary of the percentage (%) of MDA responses per group, considering the first 120 min of stimulation. The reported significances are derived from a χ^2 analysis. (*) P<0.05 and (**) P<0.01 versus controls (CTR).

7NI at several doses differently influenced MDA parameters, when compared to baseline values. In particular, in the group treated with 7NI at 50 mg/kg a within-treatment ANOVA showed a significant reduction of MDA duration ($F_{(18,156)}$ = 2.13; P= 0.0076) with a maximum effect at 110th min (D%: -53.00% ± 22.76) and of the AD duration ($F_{(18,156)}$ = 1.798; P= 0.03) maximum at 120th min (D%:-42.88 ± 24.33). Whereas, only a non-significant increase in the time of the onset was evidenced. The same analysis for 7NI at 35 and 20 mg/kg for onset, MDA and AD durations did not reveal noticeable statistical differences. Also, between-

treatments comparison on MDA parameters of 7NI at 50 mg/kg showed differences versus 7NI at 35 and 20 mg/kg in stimulus time (Onset: $F_{(17,486)}$= 1.933, P=0.0139; MDA: $F_{(17,486)}$= 1.387, P<0.0001 and AD: $F_{(17,486)}$= 1.097, P=0.3531), treatment (Onset: $F_{(17,486)}$= 42.12, P< 0.0001; MDA: $F_{(17,486)}$= 37.12, P= 0.1375 and AD: $F_{(17,486)}$= 32.08, P<0.0001) and interaction (Onset: $F_{(17,486)}$=0,8714, P=0.6786; MDA: $F_{(17,486)}$= 0.9956, P=0.4779 and AD: $F_{(17,486)}$= 0.8701, P=0.6807). Post-hoc results outlined a reduction in MDA and AD durations of 7NI 50 for P<0.05 (as indicated in fig. 10 b).

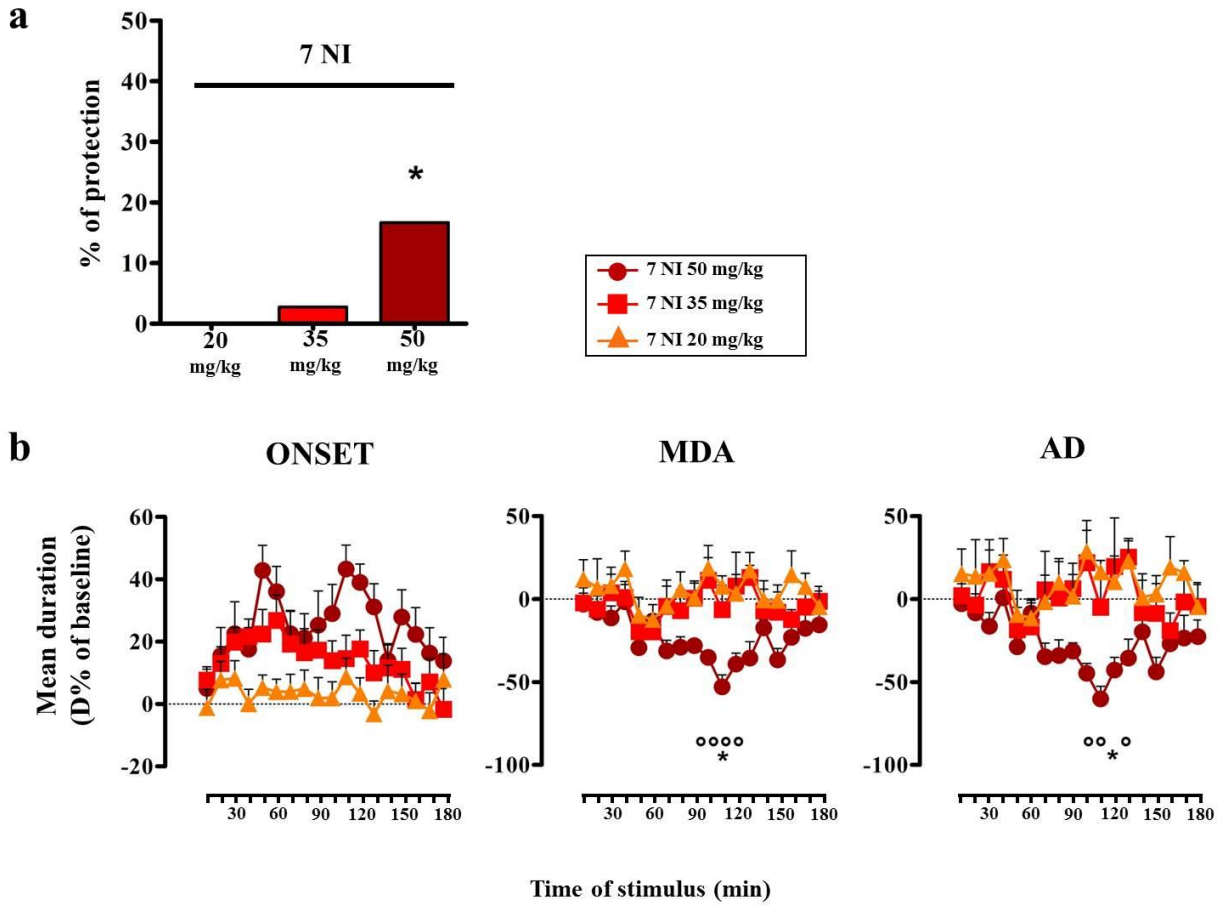

Fig. 10. *(a)* Effects of 7NI at 50, 35 and 20 mg/kg on the % of MDA protection. (*) for the experimental groups that presented significant differences at least in one stimulation time point, versus controls (for P<0.05). *(b)* Time course of MDA parameters during the 18 progressive stimuli. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (● 7NI 50 mg/kg, ■ 7NI 35 mg/kg, ▲ 7NI 20 mg/kg). Within-treatment statistically significant D% of 7NI at 50 mg/kg is indicated as (*) for P<0.05 vs baseline values. Between-treatments significance of 7NI at 50 mg/kg was indicated as (◦) for P<0.05 versus 7NI at 35 mg/kg.

Effect of 7NI and ARG on cannabinoid activity

Following single drug treatments, 7NI and WIN were co-administered at doses proved to be singularly ineffective for protection from electrically-induced seizures, i.e. 20 mg/kg for 7NI and 5 mg/kg for WIN (Tab. 3 and fig. 11 a). No changes in MDA responses were found after pretreatment with 7NI up to the time of WIN administration. The χ^2-test performed on the number of responding animals after 7NI–WIN co-treatment revealed a strong protection from seizures starting 10 min after the administration of the second drug and lasting up to 70 min, with a maximum effect between 20^{th} and 40^{th} min when only 20% of animals responded to the stimulation (χ^2=13.33, DF=1, P=0.0003). This result showed a significant 7NI–WIN-induced enhancement of protection from epileptic discharges, when compared to the outcomes of WIN at 5 mg/kg; indeed, the maximal D% of protection was observed at 20^{th} and 30^{th} min versus WIN at 5 mg/kg (D%:+70%, χ^2=9.899, DF=1, P=0.0017). The observed effect was abolished when animals were pretreated with the CB_1 antagonist AM251 before 7NI and WIN injections. MDA response were almost always elicited since only 10% of animals did not respond for a short-time interval between only at 50^{th} and 60^{th} min after WIN administration. Vehicle tests for co-treatment did not show any change in WIN 5 mg/kg single action.

As for MDA parameters, the effects of co-treatment with WIN (5 mg/Kg) and 7NI (20 mg/Kg) was analysed for within-treatment statistical differences. In particular, the time of onset significantly increased with respect to baseline values ($F_{(18,139)}$ = 1.995, P=0.0147); post-hoc Bonferroni test showed a significant increase with maximum effect at 20^{th} min (D%:+47.10 ± 4.78). Additionally, a within-treatment decrease in MDA and AD durations emerged (respectively: $F_{(18,139)}$ = 4.214, P<0.0001 and $F_{(18,139)}$ = 4.395, P<0.0001). In particular, post-hoc comparisons highlighted significant differences from 10^{th} to 130^{th} min and at 160^{th} and 170^{th} min, reaching a maximum at 50^{th} min (respectively: D%: -60.15 ± 16.85% and D%: -88.79 ± 2.29%). As for between-treatments comparisons of 7NI-WIN

versus WIN at 5mg/kg, a two-way ANOVA for the time of onset revealed significant main effects of stimulus time ($F_{(17,252)}$ = 2.31, P =0.0028), treatment ($F_{(17,252)}$ = 22.32, P<0.0001), but not their interaction ($F_{(17,252)}$ = 1.47, P=0.104). The same analysis performed on MDA showed significant main effects of stimulus time ($F_{(17,252)}$ = 2.36, P=0.0022) and treatment ($F_{(17,252)}$ = 81.91, P <0.0001), but not their interaction ($F_{(17,252)}$ = 0.57; P=0.90). Regarding AD durations a significant main effect emerged for stimulus time ($F_{(17,252)}$ = 3.15, P<0.0001), treatment ($F_{(17,252)}$ = 92.3, P <0.0001) but not their interaction ($F_{(17,252)}$ = 0.96, P=0.49). Bonferroni post-hoc test revealed that 7NI-WIN administration significantly increased onset time versus WIN at 5 mg/kg at 10^{th} and 20^{th} min, and reduced MDA and AD duration respectively at 50^{th} and 60^{th} min and from 30^{th} to 70^{th} min (P<0.05, fig. 11 b).

As observed in the analysis of % MDA protection, the presence of AM251 reduced the antiepileptic effects of 7NI-WIN co-treatment. In detail, within-treatment ANOVA on AM251-7NI-WIN showed a significant increase in onset time ($F_{(18,149)}$ = 2.30, P=0.0037) and a reduction of MDA and AD durations ($F_{(18,149)}$ = 0.30, P<0.0001and $F_{(18,149)}$ = 0.33, P< 0.0001), reaching a maximum effect at 120^{th} min (onset D%: 20.72 ±18.14; MDA D%: -27.03± 50.95 and AD D%:-25.30± 70.38). Though, in the onset time between-treatments comparison versus 7NI-WIN co-treatment alone showed significant main effects of stimulus time ($F_{(17,288)}$ = 2.66, P=0.0005), but not of treatment ($F_{(17,288)}$ = 0.07, P=3.30) and their interaction ($F_{(17,288)}$ = 1.14, P=0.31). This analysis conducted on MDA and AD durations of AM251-7NI-WIN group with respect to 7NI-WIN highlighted significant main effects of stimulus time (MDA: $F_{(17,288)}$ = 1.91, P<0.0001 and AD: $F_{(17,288)}$ = 3.17, P<0.0001) and treatment (MDA: $F_{(17,288)}$ = 177.8, P <0.0001 and AD: $F_{(17,288)}$ = 199.2, P<0.0001), but not their interaction (MDA: $F_{(17,288)}$ = 1.40, P=0.13 and AD: $F_{(17,288)}$ = 1.29, P=0.19; fig. 11 b). Indeed, between-treatments post-hoc tests revealed significant reductions of MDA (at 10^{th}, 30^{th} and 60^{th} min, and from 110^{th} to 130^{th} min) and of AD durations (at 10^{th}, 30^{th},50^{th} and 60^{th}

min, from 110th to 130th min and at 170th min) in 7NI-WIN group compared to AM251-7NI-WIN (for P<0.05) , as indicated in fig. 11 b.

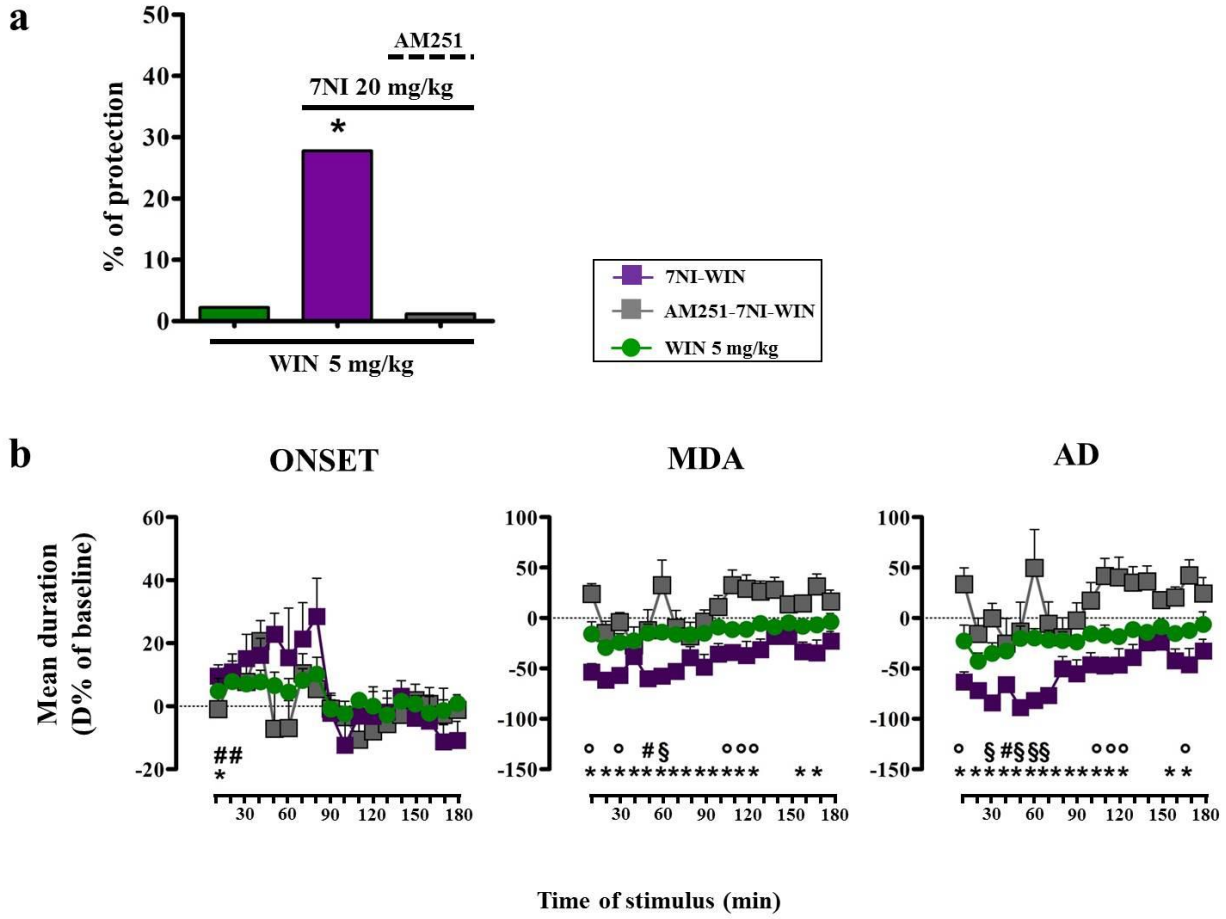

Fig. 11. *(a)* The bar graph shows the % of protection for WIN (5 mg/kg), alone and co-treated with 7NI (20 mg/kg) or with both 7NI and AM251 (1 mg/kg). (*) for the experimental groups that presented significant differences at least in one stimulation time point, versus controls (for P<0.05). *(b)* Time course of MDA parameters during the 18 progressive stimuli. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (■ 7NI-WIN, ■ AM251-7NI-WIN, ● WIN 5 mg/kg). Within-treatment statistically significant D% of 7NI-WIN is indicated as (*) for P<0.05 vs baseline values. Between-treatments significance of 7NI-WIN group is shown as (#) for P<0.05 versus WIN at 5 mg/kg; as (°) for P<0.05 versus AM251-7NI-WIN and as (§) versus both WIN at 5 mg/kg and AM251-7NI-WIN group.

Furthermore, ARG, the NO precursor, was administered before WIN at 10 mg/kg to evaluate the effects of activating nitrergic production. The % of protection from seizures (15%) was

reduced compared to WIN alone, and only a non-significant reduction of responses was observed between 30th and 50th min (Tab. 3 and fig. 12 a). Within-treatment analyses on MDA parameters showed no significant effects of ARG-WIN versus baseline values. Also, between-treatments comparison with WIN alone on MDA parameters highlighted significant main effects of treatment (Onset: $F_{(17,180)} = 67.64$, $P<0.0001$; MDA: $F_{(17,180)} = 41.36$, $P<0.0001$ and AD: $F_{(17,180)} = 29.15$, $P<0.0001$) and interaction (Onset: $F_{(17,180)} =2.54$, $P=0.012$; MDA: $F_{(17,180)} = 2.82$, $P= 0.0003$ and AD: $F_{(17,180)} =2.67$, $P=0.0006$), but not of stimulus time (Onset: $F_{(17,180)} = 1.9$, $P=0.002$; MDA: $F_{(17,180)} = 1.58$, $P=0.07$ and AD: $F_{(17,180)} = 1.59$, $P=0.069$). Post-hoc results show a reduction in the onset time in ARG-WIN from 30th to 60th min versus WIN ($P<0.05$) and an increase in MDA and AD durations from 40th to 60th min (Fig. 12 b).

Lastly, WIN at the highest dose was co-administered with ODQ to assess the implication of sGC in cannabinoid effects. A within-treatment χ^2 test revealed that in ODQ-WIN group 75% of the animals were protected from seizures from 10th to 120th min, with a maximal effect of −83.3% recorded in the stimulations where the number of responses was 1 ($\chi^2= 8.571$, DF = 1, $P<0.01$). A further comparison of the % responses in ODQ-WIN group with respect to WIN alone showed significantly reduced responsiveness at 10th, 70th and 80th min (the reduction observed was respectively: 66.6%; $\chi^2 = 6.000$, DF = 1, $P=0.0143$ at 10th min; $\chi^2 = 5.33$, DF = 1, $P=0.02$ at 70th and 80th min; tab. 3). The analysis on MDA parameters revealed that the co-treatment did not induce any significant differences in the onset parameter when compared to baseline period, whereas a reduction of MDA duration was found for the entire period of the observation, with a maximum at 90th min (D%: -75.18% ± 22.10; $F_{(15,19)} = 1.653$ and $P=0.0053$); the co-administration also reduced AD duration for 120 min, with a maximum effect at 90th min (D%:-80.40% ± 14.1; $F_{(15,19)} = 1.252$ and $P=0.0130$). Due to the massive ODQ-WIN-induced reduction of responses to stimulation (n= 1 at various stimulation time

points), a between-treatments analysis to compare ODQ-WIN effects to WIN administration alone on the MDA parameters did not allow any statistical comparisons.

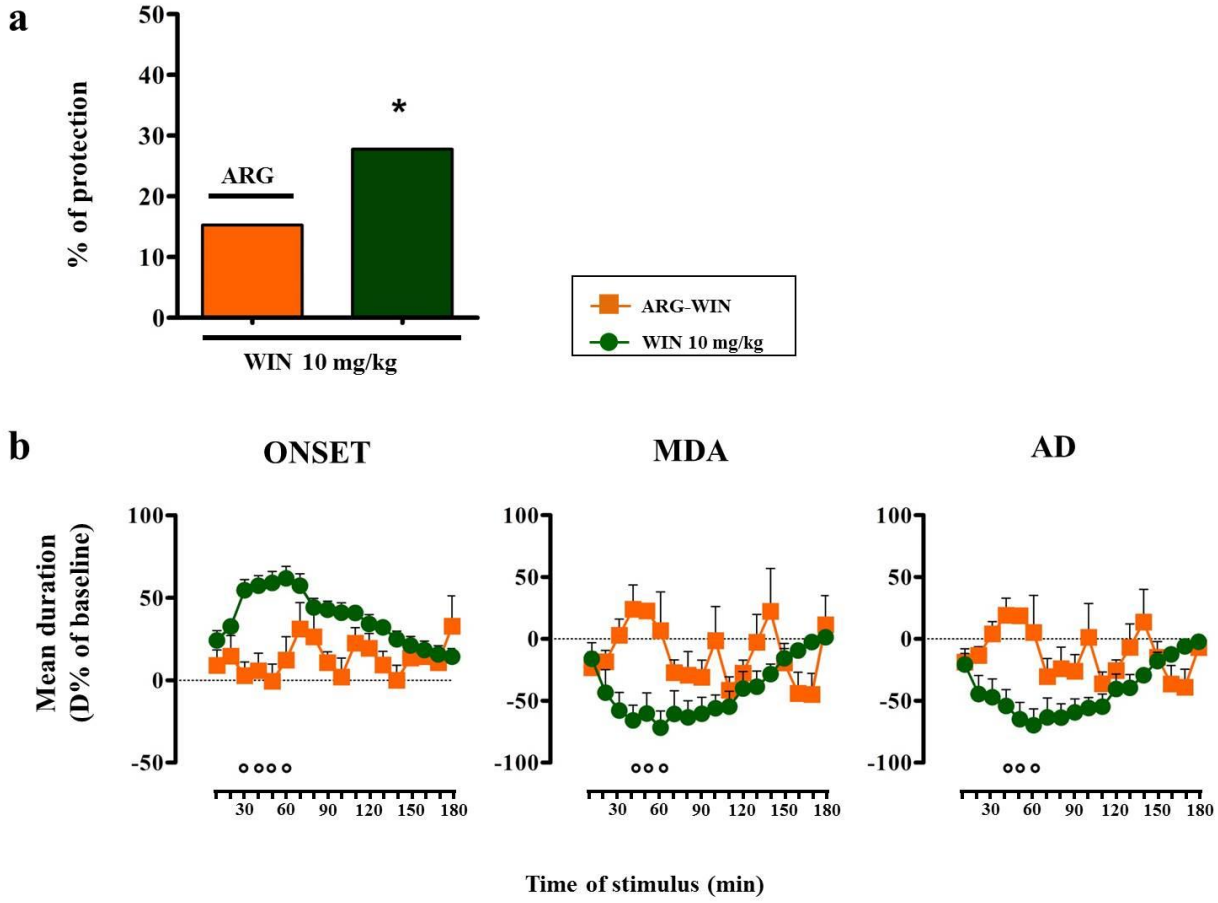

Fig. 12. *(a)* The bar graph shows the % of protection for WIN (10 mg/kg), alone and co-treated with ARG. (*) for the experimental groups that presented significant differences at least in one stimulation time point, versus controls (for P<0.05). *(b)* Time course of MDA parameters during the 18 progressive stimuli. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (■ ARG-WIN, ● WIN 10 mg/kg). Between-treatments significance of WIN at 10 mg/kg was indicated as (°) for P < 0.05 versus ARG-WIN.

Pilocarpine model

Control and vehicle-treated groups

Both control and vehicle-treated groups showed severe generalized seizures and no survival was recorded after the experimental sessions (Tab. 4 and fig. 13).

Group	Severe seizures %	Mortality (24h) %
CTR	100	100
WIN 10 mg/kg	0***	0***
WIN 5 mg/kg	0***	20**
WIN 1 mg/kg	90	80
7NI 50 mg/kg	0***	50**
7NI 35 mg/kg	0***	60*
7NI 20 mg/kg	70	80
7NI-WIN	0***	50**
AM251-7NI-WIN	80	80

Tab. 4. Summary of the percentage (%) of severe seizures and mortality (24 h) in the pilocarpine model per group. A χ^2 test was performed for statistical comparisons. (*) for P<0.05; (**) P<0.01; (***) P<0.001 versus controls (CTR). Doses for 7NI–WIN and AM251-7NI–WIN groups are indicated in the text.

Fig. 13. Examples of behavioural symptoms of increasing severity, showed by vehicle-treated rat after pilocarpine injection.

Effect of single doses of WIN and 7NI on % of severe seizures and mortality

A χ^2 analysis performed for the percentage of animals with severe seizures in WIN-treated groups showed a similar reduction for both WIN at 10 and 5 mg/kg (0%, $P<0.001$) with respect to controls, whereas WIN 1 mg/kg did not show statistical differences (severe seizures: 90%; tab. 4). In addition, statistical comparisons of mortality rates revealed that WIN 10 mg/kg dampened percentages of mortality (all survived, $P<0.001$) versus controls, as well as WIN 5 mg/kg (20% mortality, $P<0.01$), while WIN 1 mg/kg did not significantly modify this parameter (mortality rate: 80%). Furthermore, an evaluation of CB_1 receptors role on anticonvulsant effect of WIN was carried out administering the CB_1R antagonist AM251 before the most effective dose of WIN (10 mg/kg). With this approach, WIN effects were impaired and % of severe seizures went up (90%, $P=0.0001$) as well as mortality (60%, $P=0.0034$) versus WIN 10 mg/kg group.

As for 7NI effect on the percentage of severe seizures, after 7NI at both 50 and 35 mg/kg the amount of animals reaching score 5 seizures was dropped with respect to controls (0%, $P<0.001$), while after 7NI at 20 mg/kg significant changes were not observed (severe seizures: 70%). Similar analyses performed on the mortality rate revealed that only 7NI at both 50 and 35 mg/kg significantly reduced the percentages of dead animals within 24h from the experimental session. 7NI at 50 mg/kg showed 50% mortality ($P<0.01$) and 7NI at 35 mg/kg 60% mortality ($P<0.05$), respectively analysed versus the 100% mortality of controls.

Effect of single doses of WIN and 7NI on behavioural scoring and latency to the first seizure state

Results of a Kruskal–Wallis test, performed on behavioural scoring for WIN-treated groups, including ‘‘treatment’’ as the between-groups factor, revealed statistical differences between

groups (P=0.0003). Post-hoc analysis highlighted significantly reduced behavioural symptoms in WIN 10 mg/kg (total scoring of 0.65±0.75, P<0.0001) and 5 mg/kg (1.3±0.34, P<0.05) groups with respect to controls, but behavioural scoring was not significantly different in WIN 1 mg/kg versus controls (2.09±0.4; fig. 14 a). Significant changes were found in latencies of pilocarpine-induced behavioural alterations for WIN groups with respect to controls ($F_{(3,36)}$ = 3.764, P=0.019).

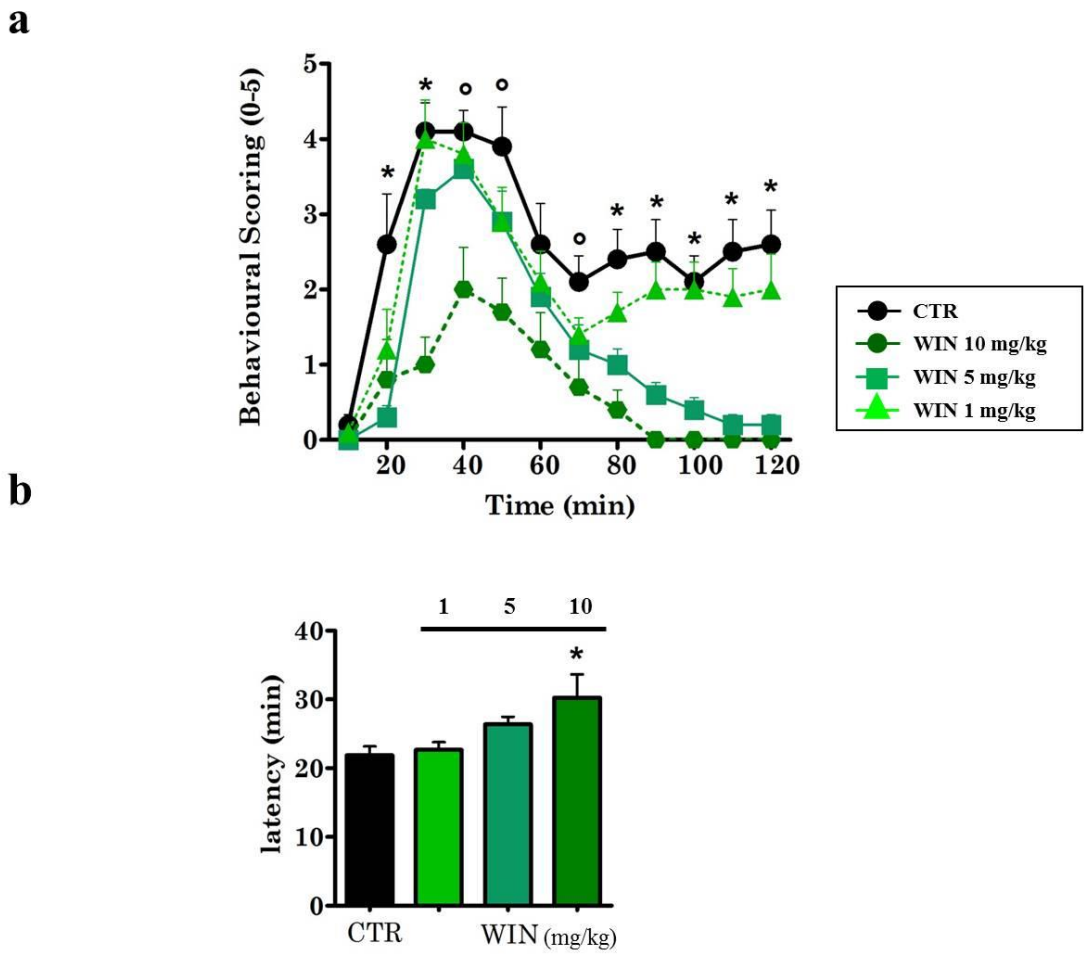

Fig. 14. *(a)* Effects of WIN (10, 5, 1 mg/kg) on behavioural scores during the 120 min following the administration of pilocarpine. Fisher's exact test per time point evidenced significant differences as described hereafter. (°) for P<0.05 of WIN (10 mg/kg) versus controls (CTR); (*) for P<0.05 of both WIN at 10 and 5 mg/kg respectively versus CTR. *(b)* Latency in min to first seizure state. A within-treatment one-way ANOVA evidenced statistically significant differences for (*) P<0.05 versus control group. All values are presented as mean ± S.D.

In particular, post-hoc comparisons highlighted a marked increase only in the latency of WIN at 10 mg/kg versus controls (from 21.88±4.08 of CTR to 30.26±10.73 min of WIN 10 mg/kg, P<0.05; fig. 14 b). As for an aspect considered in other research (Wallace et al., 2003), at the maximal used dose of WIN (10 mg/kg), animals showed mild sedation, especially in the last 30 min of observation, without any locomotor alterations. On the other hand, with WIN at 5 mg/kg rats were not evidently affected and alert enough to be able to move freely. WIN 1 mg/kg did not induce any aversive signs.

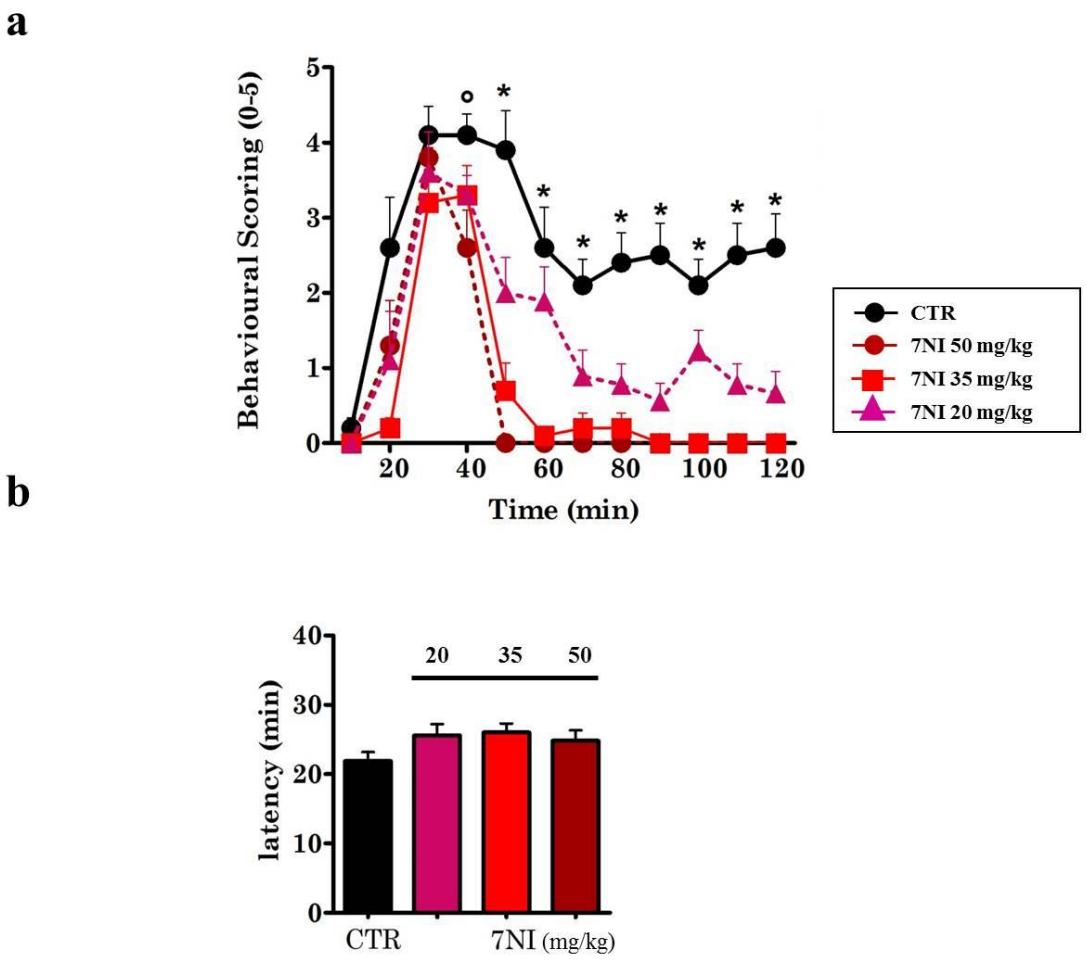

Fig. 15. *(a)* Effects of 7NI (20, 35 and 50 mg/kg) on behavioural scores during the 120 min following the administration of pilocarpine. Fisher's exact test per time point evidenced significant differences as described hereafter. (°) for P<0.05 of 7NI (50 mg/kg) versus controls (CTR); (*) for P<0.05 of both

7NI at 50 and 35 mg/kg, respectively versus CTR. *(b)* Effect of 7NI at different doses on latency in min to first seizure state. All values are presented as mean ± S.D.

The same analysis on the effect of 7NI on total behavioural score showed significant differences among the experimental groups (P=0.0004). Dunn's post-hoc analysis highlighted a significantly reduced behavioural score in 7NI at both 50 mg/kg (total scoring of 0.64±0.67, P<0.0001) and 35 mg/kg (0.66±0.51, P<0.001), respectively versus controls (total scoring of 2.64±0.43); behavioural scoring of rats treated with 7NI 20 mg/kg was non-statistically different than controls (Fig. 15 a). Non-significant changes were found in the latency of pilocarpine-induced behavioural alterations for all 7NI groups with respect to controls (Fig. 15 b).

As for the involvement of CB_1 receptors in WIN effects, when AM251 was administered before the highest dose of WIN, the behavioural scoring revealed statistical differences between groups (P=0.0007), with an increase in total scoring of 1.34±0.5 for AM251-WIN group with respect to WIN 10 mg/kg alone. Fisher's exact test showed that the effect of AM251 was maintained up to 110^{th} min (for P<0.05). As regards the analysis of latency, a one-way ANOVA revealed an early effect of AM251 since significant differences emerged between AM251-WIN, WIN 10 mg/kg and control groups ($F_{(2,27)}$ =8.65; P=0.012). In detail, post-hoc analysis highlighted that latency of AM251-WIN group was significantly decreased compared to WIN 10 mg/kg (from 30.26±10.73 of WIN 10 mg/kg to 17.30±4.21 min of AM251-WIN, P<0.001).

Effect of 7NI–WIN and AM251-7NI–WIN groups on % severe seizures and mortality

The 7NI–WIN co-treatment respectively with 20 mg/kg and 1 mg/kg, singularly ineffective doses, did not elicit any severe seizures (0%, P<0.0001) and decreased the mortality by 50%

(P<0.01), as shown by χ^2-test statistical analysis versus controls (Tab. 4). When animals were pretreated with the CB1 antagonist AM251, before 7NI and WIN injections, 7NI–WIN effects were impaired and % of severe seizures went up to 80% (P=0.0003) as well as mortality (80%, P=0.022) versus 7NI–WIN group.

Effect of 7NI–WIN and AM251-7NI–WIN groups on behavioural scoring and latency to the first seizure state

Analysis to evaluate the effect of 7NI–WIN co-treatment revealed statistical differences on the behavioural scoring between groups (P=0.0109). Particularly, a post-hoc analysis showed a significant reduction of behavioural scores in 7NI–WIN group with respect to WIN alone at 1 mg/kg (total scoring of 0.8±0.76, P<0.01; fig. 16 a). Whereas, this protective outcome is no more obtained when CB_1 receptors are antagonized with AM251 (total scoring of 1.77±0.56) when compared to 7NI–WIN and WIN 1 mg/kg. The behavioural scoring in AM251-7NI–WIN appears higher than 7NI–WIN group for the 120-min observation (Fig. 16 a). As for analysis of latency, a one-way ANOVA revealed significant differences ($F_{(3,36)}$ = 9.039; P=0.0001). In detail, post-hoc analysis outlined that latency of 7NI–WIN group was significantly increased compared to WIN 1 mg/kg (from 22.67±3.55 of WIN 1 mg/kg to 31.95±3.26 min of 7NI–WIN, P<0.001; fig. 16 b). Furthermore, the pretreatment with AM251 significantly averted 7NI–WIN effects on latency (24.66±7.29 min of AM251-7NI–WIN group, P<0.05; fig. 16 b). Vehicle tests for co-treatment did not show any change in WIN 1 mg/kg single action. Lastly, no WIN-associated side effects were observed in the 7NI–WIN co-treatment.

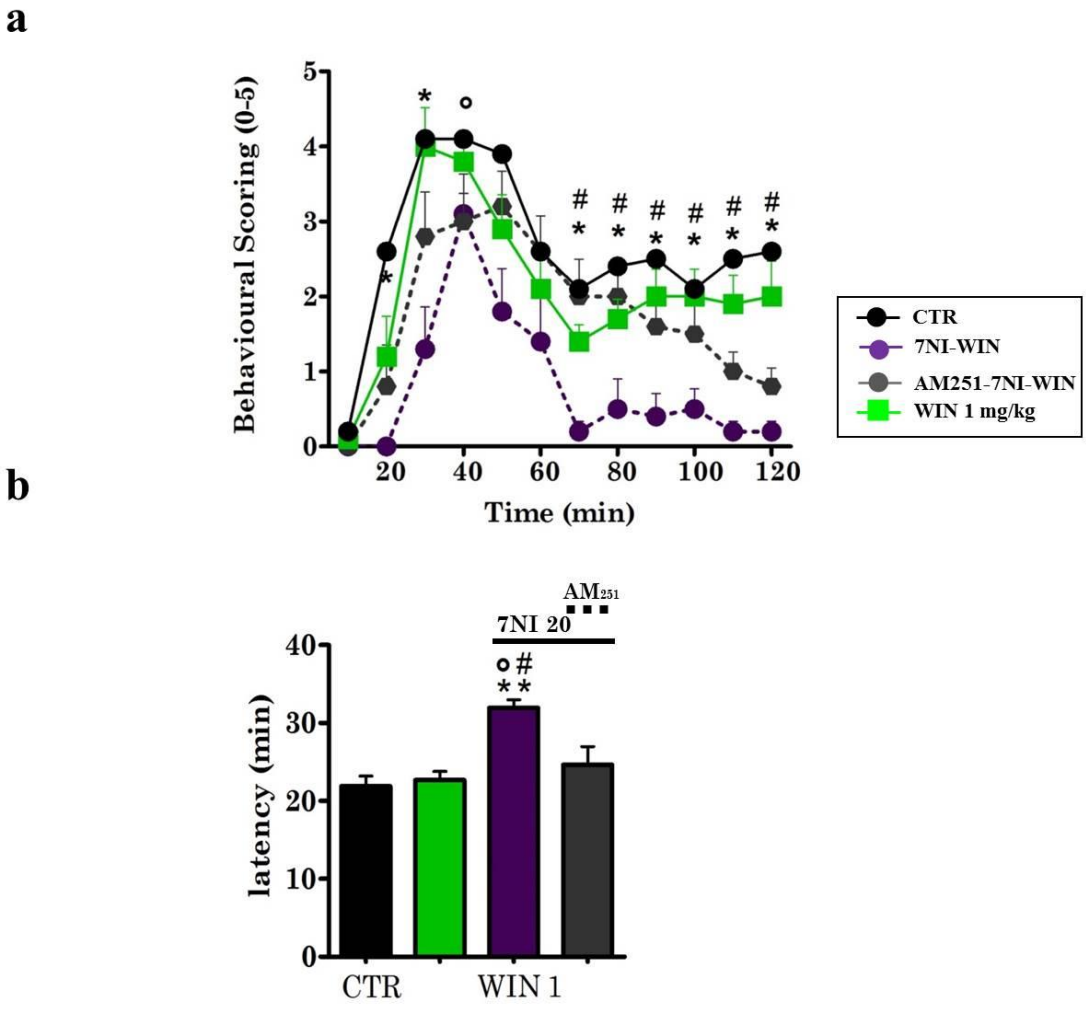

Fig. 16. *(a)* Behavioural scores during the 120 min following the administration of pilocarpine. Results of Fisher's exact test evidenced significant differences as described. (°) for P<0.05 of 7NI–WIN versus controls (CTR); (*) for P<0.05 of 7NI–WIN, respectively versus WIN (1 mg/kg) and controls; (#) for P<0.05 of 7NI–WIN versus AM251-7NI–WIN. *(b)* Latency to first seizure state. P<0.001 (**) versus control group. A between-treatments analysis was performed for the 7NI–WIN group and revealed significant differences indicated with (°) for P<0.05 versus WIN (1 mg/kg) and with (#) for P<0.05 versus AM251-7NI–WIN groups. All values are presented as mean ± S.D.

EXPERIMENT 2

MDA model

Time of stimulus	CAP-WIN	CPZ-WIN 5 mg/kg	CPZ-WIN 10 mg/kg	7NI-CAP-WIN	ARG-CAP-WIN
10	100	100	67	67	67
20	33**	83	33**	33**	50*
30	83	67	33**	33**	83
40	83	83	33**	67	83
50	100	100	33**	67	100
60	100	100	67	83	100
70	100	100	50*	83	100
80	100	100	50*	83	100
90	100	100	50*	100	100
100	100	100	50*	100	100
110	100	100	67	100	100
120	100	100	67	100	100

Tab. 5. Summary of the percentage (%) of MDA responses per group, considering the first 120 min of stimulation. The reported significances are derived from a χ^2 analysis. (*) P<0.05. (**) P<0.01 versus controls (CTR). Values for CAP and CPZ at different doses were not included in table since these drugs always induced 100% MDA responses.

Effect of single doses of capsaicin on % of responses and MDA parameters

The effects of capsaicin were evaluated using distinct doses in separate groups: 1, 5 or 10 mg/kg i.p. The % of protection was not modified with respect to controls; indeed, all animals treated with CAP at different dosages responded to AB stimulation (% protection = 0). Furthermore, a within-treatment ANOVA was used to compare possible changes of MDA parameters with baseline values. The treatment with CAP at 10 mg/kg induced significant differences (MDA duration, $F_{(18,92)} = 2.06$, P=0.0135; AD duration, $F_{(18,92)} = 2.05$, P=0.014; fig. 17 a). Post-hoc analysis revealed that the time of onset was significantly reduced from 40^{th} to 140^{th} min with a maximum effect at 110^{th} min (D% = −24.98 ± 22.42), whilst MDA

and AD durations were statistically increased from 20th to 140th stimulation with a maximum effect at 90th min (D% = +102.26 ± 49.01 and +173.10 ± 97.88, respectively). In contrast, analyses on the effects of lower dosages of CAP showed no statistical differences compared with baseline. Lastly, a between-treatments ANOVA was performed to compare the effects of different doses of CAP. No changes were observed for the time of onset comparing CAP at 10 mg/kg with CAP at 5 and 1 mg/kg and controls. Instead, the duration of MDA and AD in CAP 10-treated animals was significantly increased with respect to CAP at 5, 1 mg/kg and controls (for $P<0.05$; fig. 17 a and as represented in fig. 7 d).

Effect of single doses of capsazepine on % of responses and MDA parameters

Three drug doses (0.5, 1 or 2 mg/kg, i.p.) were administered in order to evaluate the effects of capsazepine in different groups. All animals treated with CPZ responded to AB stimulation (% of protection = 0). Then, a within-subject analysis was performed for MDA parameters. CPZ at 2 mg/kg was able to significantly increase the duration of time of onset ($F_{(18,95)} = 2.35$, $P = 0.0041$) as well to reduce MDA ($F_{(18,95)} = 3.47$, $P<0.0001$) and AD ($F_{(18,95)} = 3.47$, $P<0.0001$; fig. 17 b) durations. Post-hoc test showed that these effects were distributed from 20th to 160th min with a maximum effect at 100th min (D% onset = +31.54 ± 11.36; D% MDA duration = −42.95 ± 2.77; D% AD duration = −64.81 ± 7.94). The administration of CPZ at 1 and 0.5 mg/kg did not induce any changes in MDA parameters when compared to baseline values. As for between-treatments comparisons, CPZ at 2 mg/kg significantly increased onset duration when compared to CPZ at 0.5 mg/kg and controls, but not versus CPZ at 1 mg/kg. Furthermore, the duration of MDA and AD in_CPZ 2 mg/kg was statistically reduced with respect to CPZ at 0.5 and 1 mg/kg and controls (for $P<0.05$; fig. 17 b).

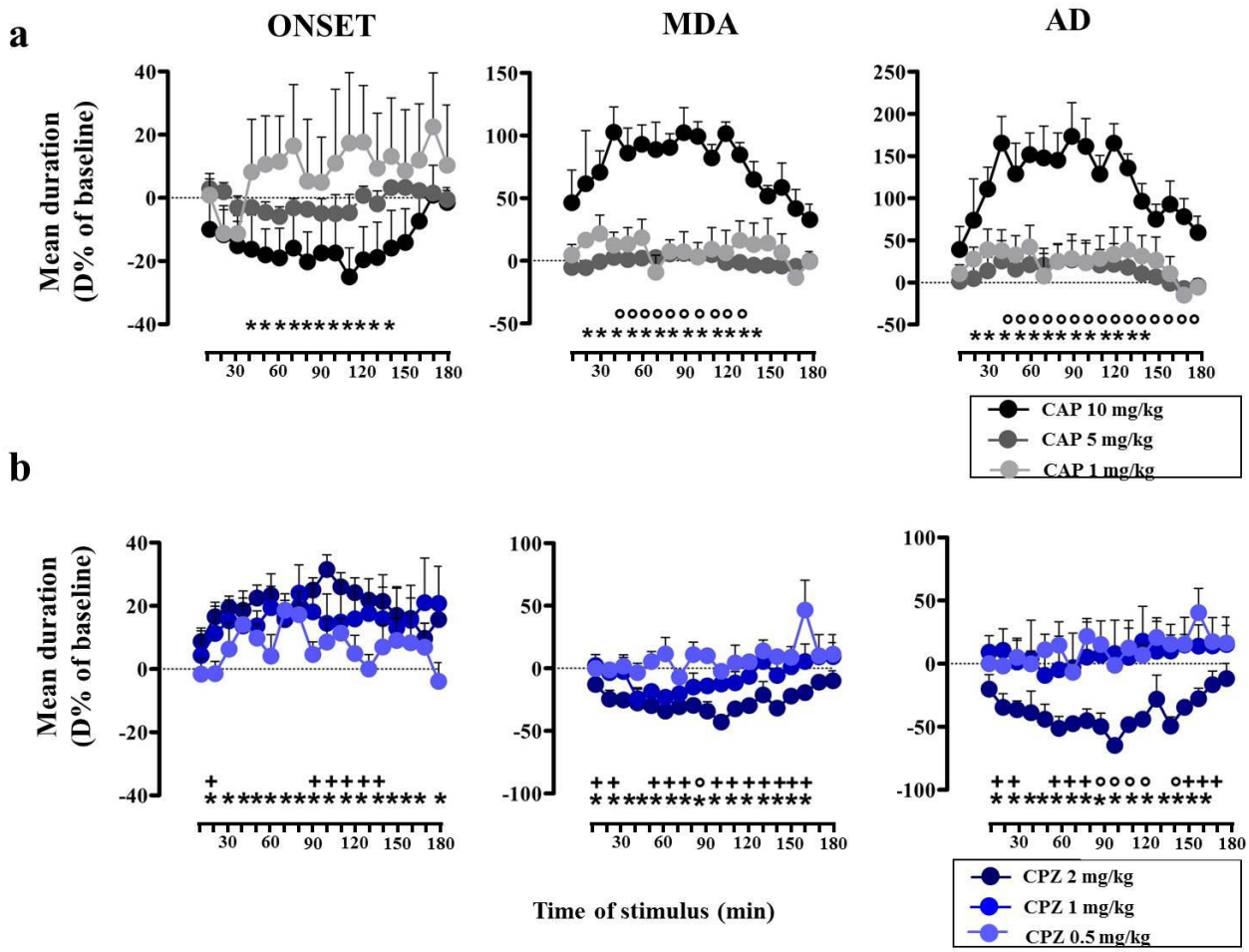

Fig. 17. *(a)* Effects of CAP at 10, 5 and 1 mg/kg on the time course of MDA parameters during the 18 progressive stimuli. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (● CAP 10 mg/kg, ● CAP 5 mg/kg, ● CAP 1 mg/kg). Within-treatment statistically significant D% of CAP at 10 mg/kg is indicated as (*) for P<0.05 vs baseline values. Between-treatments significance of CAP at 10 mg/kg was indicated as (◦) for P<0.05 versus CAP 5 and 1 mg/kg. *(b)* Effects of CPZ at 2, 1 and 0.5 mg/kg on MDA parameters during 18 stimuli. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (● CPZ 2 mg/kg, ● CPZ 1 mg/kg, ● CPZ 0.5 mg/kg). Within-treatment statistically significant D% of CPZ at 2 mg/kg is indicated as (*) for P<0.05 versus baseline values. Between-treatments significance of CPZ 2 mg/kg was indicated as (◦) for P<0.05 versus CPZ 1 and 0.5 mg/kg, and as (+) for P<0.05 only versus CPZ 0.5 mg/kg.

Effect of capsaicin on % of responses and MDA parameters of rats treated with WIN at 10 mg/kg

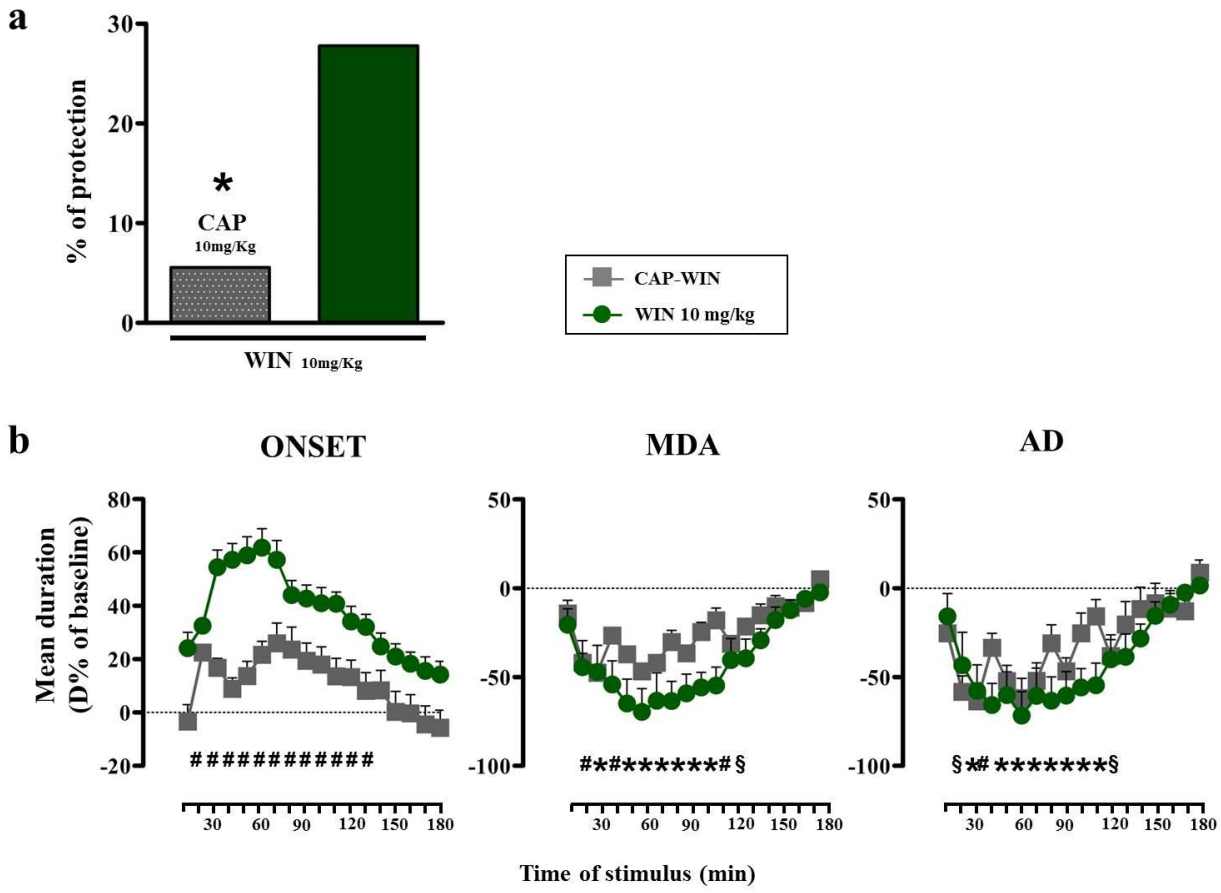

Fig. 18. *(a)* Effects of co-treatment with CAP at 10 mg/kg and WIN at 10 mg/kg on % MDA protection versus WIN at 10 mg/kg. *(b)* Effects of co-treatment on MDA parameters during 18 stimuli compared with WIN at 10 mg/kg. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (■ CAP-WIN, ● WIN 10 mg/kg). Within-treatment statistically significant D% is indicated for $P < 0.05$ (* for both groups, § for co-treatment, # for WIN group) versus baseline values.

When animals were co-treated with both CAP at 10 mg/kg and the WIN dose of 10 mg/kg, the % of protection was reduced when compared to WIN 10 mg/kg (Fig. 18 a) and the duration of this protection was limited from 20th to 40th min, but significant only at 20th min when 33.33% of animals responded to AB stimulation ($\chi^2 = 6.00$, DF = 1, P=0.0014) (Tab. 5).

Furthermore, a within-treatment analysis showed that the co-treatment with CAP and WIN induced significant changes on MDA parameters with respect to baseline. The time of onset was not statistically different, but significant reductions were found for MDA ($F_{(18,89)}$ = 2.89, P=0.0005) and AD ($F_{(18,89)}$ = 4.0, P=0.0001). Post-hoc tests showed that MDA was decreased from 30th to 120th min, with a maximum effect at 30th min (D% = −47.57 ± 6.31); while AD was reduced from 20th to 120th min with a maximum effect at 30th min (D% = −63.67 ± 25.30). Between-treatments comparisons were performed between the co-treated group versus WIN 10 and controls (Fig. 18 b). Though, for all parameters it is evident a weaker effect of the co-administration of CAP and WIN with respect to WIN alone, the between-treatments analysis did not reach statistical significance. However, the co-treated group maintains a significant antiepileptic effect when compared to controls.

Effect of capsazepine on % of responses and MDA parameters of rats treated with WIN at 10 mg/kg

The co-administration of CPZ at 2 mg/kg and WIN at 10 mg/kg noticeably enhanced the % of protection with respect to WIN 10 mg/kg (Tab. 5 and fig. 19 a). Indeed, a clear protection was found from 20th to 140th min, with significant values up to 110th min and a maximal effect at 20th to 50th interval when only 33.3% of animals responded to AB stimulation (χ^2= 6.00, DF = 1, P=0.0014). Moreover, a within-treatment analysis on MDA parameters revealed that there was both a significant increase in the onset time ($F_{(18,56)}$ = 6.01, P<0.0001) and a reduction in MDA ($F_{(18,56)}$ = 11.69, P<0.0001) and AD ($F_{(18,56)}$ = 7.06, P<0.0001), when compared to baseline. Post-hoc tests showed that onset was increased from 10th to 110th min, with a maximal effect at 30th min (D% = +68.33 ± 30.64); MDA and AD went down from 10th to 140th min with a maximum effect at 60th min (D%(D% = −77.36 ± 10.87) and at 20th min (D% = −99.09 ± 1.29), respectively. Between-treatments comparisons were performed on co-

treated group with respect to WIN 10 mg/kg and controls (Fig. 19 b). Analyses on differences versus WIN 10 mg/kg showed no statistical changes on MDA parameters. Lastly, the co-treated group shows a marked antiepileptic effect when compared to controls.

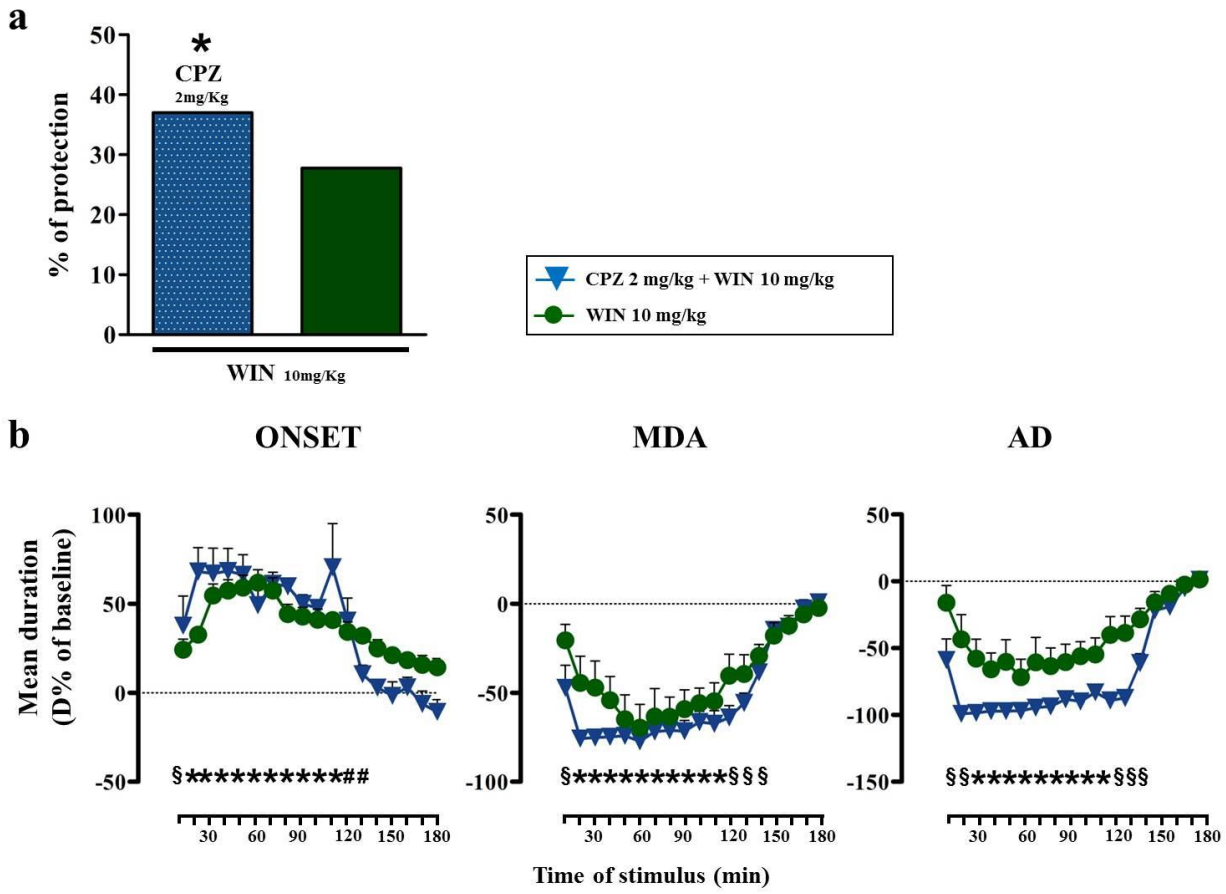

Fig. 19. *(a)* Effects of co-treatment with CPZ at 2 mg/kg and WIN at 10 mg/kg on % MDA protection versus WIN at 10 mg/kg. *(b)* Effects of co-treatment on MDA parameters during 18 stimuli compared with WIN at 10 mg/kg. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (▼ CPZ 2 mg/kg and WIN 10 mg/kg, ● WIN 10 mg/kg). Within-treatment statistically significant D% is indicated for $P<0.05$ (* for both groups, § for co-treatment, # for WIN group) versus baseline values.

Effect of capsazepine on % of responses and MDA parameters of rats treated with WIN at 5 mg/kg

CPZ was also administered with the subeffective dose of WIN. Pre-treatment with CPZ at 2 mg/kg of animals treated with WIN at 5 mg/kg, did not modify the % of protection with respect to WIN 5 mg/kg, but significantly influenced MDA parameters (Table 5 and fig. 20 a). Indeed, the duration of the onset was significantly increased ($F_{(18,89)}$ = 2.54, P=0.0021), and reductions were found for MDA ($F_{(18,89)}$ = 5.9, P<0.0001) and AD ($F_{(18,89)}$ = 5.9, P<0.0001). Post-hoc tests highlighted that onset was changed from 40th to 60th and from 90th to 130th min with a maximum effect at 40th min (D% = +40.21 ± 29.83); while MDA and AD from 10th to 170th min with a maximum effect at 40th min (D% = −47.84 ± 7.61) and at 60th min (D% = −74.06 ± 10.69), respectively.

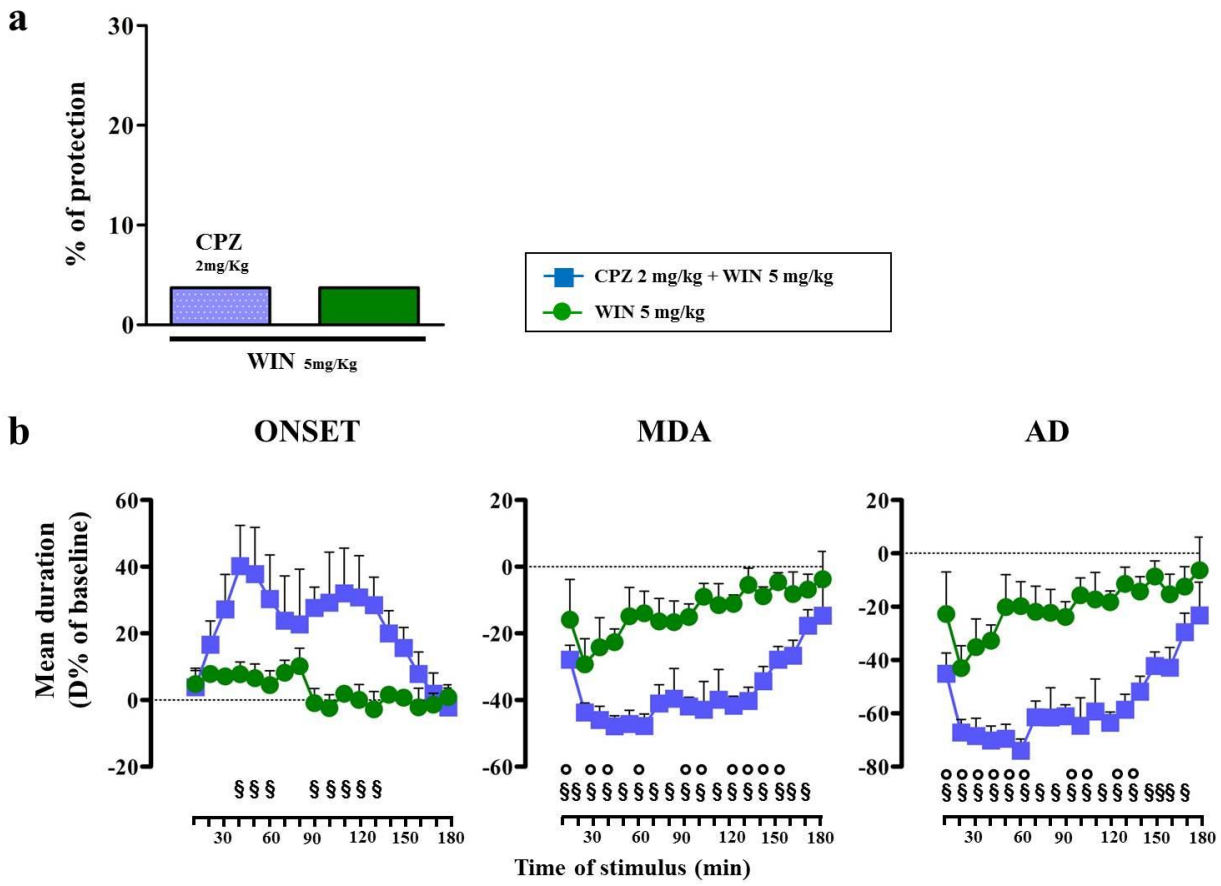

Fig. 20. *(a)* Effects of co-treatment with CPZ at 2 mg/kg and WIN at 5 mg/kg on % MDA protection versus WIN at 5 mg/kg. *(b)* Effects of co-treatment on MDA parameters during 18 stimuli compared with WIN at 5 mg/kg. Each value represents the mean of D% ± S.D. of each treatment per stimulus versus baseline values (■ CPZ 2 mg/kg and WIN 5 mg/kg, ● WIN 5 mg/kg). Within-treatment statistically significant D% is indicated for P<0.05 (§ for co-treatment) versus baseline values. Between-treatments significance of co-treated group was indicated as (◦) for P<0.05 versus WIN 5 mg/kg.

Between-treatments analyses were conducted on CPZ co-administered with WIN at 5 mg/kg versus WIN 5 mg/kg, CPZ at 2 mg/kg and controls. Significant changes were found for MDA and AD durations versus the ineffective WIN dose of 5 mg/kg (for P < 0.05). Lastly, CPZ–WIN showed significant reductions of MDA and AD duration at 30th and 120th min versus CPZ at 2 mg/kg (for P<0.05; fig. 20 b).

Effect of capsaicin on % of responses and MDA parameters of rats treated with WIN at 10 mg/kg after 7NI or ARG administration

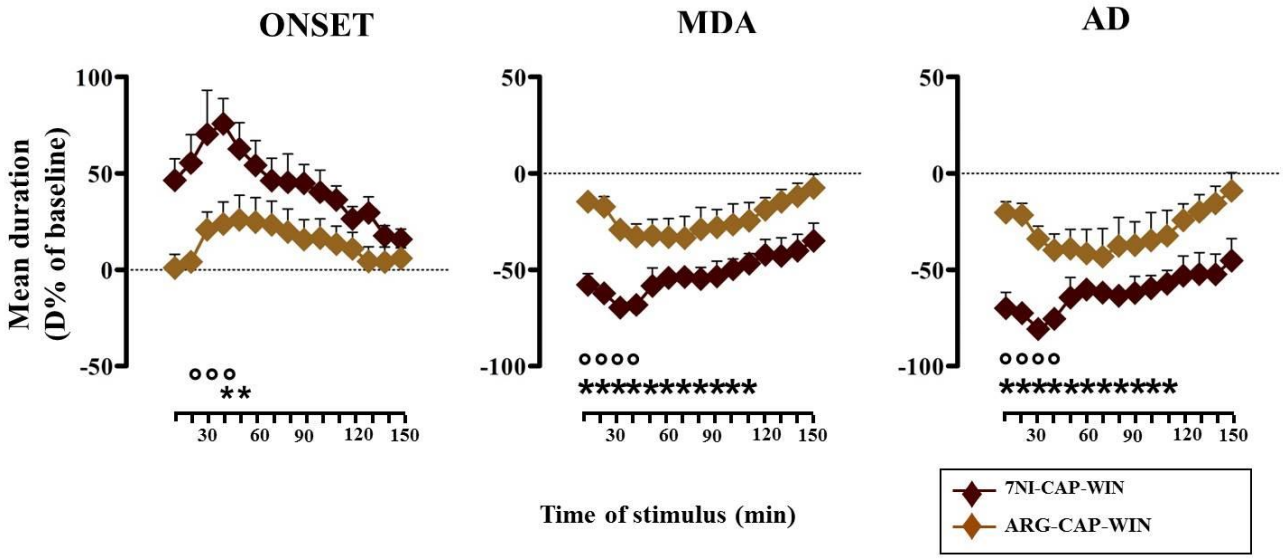

Fig. 21. Effects of 7NI-CAP-WIN and ARG-CAP-WIN groups on the time course of MDA parameters during the 15 progressive stimuli. Each value represents the mean of D% of each treatment (♦7NI-CAP-WIN or ♦ ARG-CAP-WIN) per stimulus versus baseline values. Within-treatment statistically significant D% of 7NI-CAP-WIN is indicated for P<0.05 (*) vs baseline values. Between-

treatments significance of 7NI-CAP-WIN group was indicated as (◦) for P<0.05 versus ARG-CAP-WIN.

The co-treatment of 7NI and CAP on WIN reduced % MDA response from 10^{th} to 80^{th} min versus vehicle-treated controls, reaching significance at the 20^{th} and 30^{th} (MDA responses = 33%; χ^2= 6.000, DF= 1, P=0.0143; tab. 5). As for MDA parameters, within-treatment analysis showed a significant increase in time of onset ($F_{(15,63)}$ = 1.877, P=0.043) and a decrease in MDA and AD durations ($F_{(15,63)}$ = 2.21, P=0.0146 and $F_{(15,63)}$ = 1.856; P= 0.045, respectively; fig.21). Post-hoc comparisons outlined that the increase in the onset was maximal at 40^{th} min (D%= 75.59 ± 32.23); while the reductions in MDA and AD were framed between the 10^{th} and the 110^{th} with a maximum effect at the 30^{th} (D%= -69.67 ± 5.05 and D%= -80.76 ± 11. 77, respectively; fig.21).

The administration of ARG before CAP and WIN reduced the number of responding animals from 10^{th} to 40^{th} min versus vehicle-treated controls, but a significant change was induced only at the 20^{th} (MDA responses = 50%; χ^2= 4.000, DF= 1, P=0.0045; tab. 5). Though the time course of onset seemed to have increased while MDA and AD durations were reduced, analysis on MDA parameters did not show any significant difference versus related baseline values (Fig.21). Between-treatments significant differences were found comparing 7NI-CAP-WIN with ARG-CAP-WIN groups. The time of onset in 7NI-CAP-WIN group was increased from 20^{th} to 40^{th} min and MDA and AD durations were lowered ranging from the 10^{th} to 40^{th} (P<0.05, fig.21).

DISCUSSION

Epileptic seizures originate from mechanisms altering the balance between excitation and inhibition of neuronal transmission. Electrical bases governing synaptic processes are thus fundamental aspects to consider. Animal research is crucial for investigating on synaptic mechanisms involved in seizures, giving opportunities to advance our understanding of epileptogenesis and hence the potential to treat. The most common epilepsy models elicit seizures in adult male rodents, via injection of a proconvulsant drug (i.e. pilocarpine and kainic acid) or electrical stimulation (Löscher, 2011). Administration of anticonvulsants after the onset can limit the occurring widespread damage to the brain, similarly as observed in humans (Scharfman et al., 2007). A direct consequence is that employing a double *in vivo* experimental approach could pose solid ground for determination of possible causes and individuate specific therapeutic improvements. In this view, in the present study the data were obtained by employing two models of TLE that are considered as a powerful tool to predict the response to new antiepileptic treatments (Banach et al., 2011; Curia et al., 2008). These models determine acute seizure events in naïve animals, due to different epileptogenic mechanisms respectively based on electrical stimulation and chemical triggering, in order to explore fundamental aspects of paroxysmal discharge underlying symptomatic epileptic state.

Insight into cannabinoid contribution to epileptic seizures

This research provides an insight into the contribution of cannabinoid-mediated synaptic mechanisms in rat models of epileptic seizures. Preclinical testing of cannabinoids in the last decades have boosted interest on their pharmacological power in epilepsy (Ligresti et al., 2016; Pertwee, 2012; Howlett et al., 2004; Wallace et al., 2001), despite therapeutic application may expose to acute and chronic side effects (Hill et al., 2012; Gerra et al., 2010).

However, the exact molecular mechanisms of exogenous and endogenous cannabinoids in the complex regulation of normal and paroxysmal neuronal excitability remains elusive.

In my experiments, synthetic CB agonist WIN was tested, as an ideal candidate to explore the outcomes of pharmacological activation of endocannabinoid system, since it has been used in numerous models of epileptic seizures and also its antiepileptic effect was found to go beyond CB_1-dependence in models of temporal lobe epilepsy (Suleymanova et al., 2016; Payandehmer et al., 2015; Hill et al., 2013; Rizzo et al., 2009; Jones et al., 2012, Wallace et al., 2003). The present results showed that WIN administration at increasing dosages exerts a dose-dependent protection. In that, WIN at 10 mg/kg markedly dampened paroxysmal responses along the entire observation period, while WIN at 5 mg/kg action was scarcely effective and of short duration (within 40 minutes post-treatment) and WIN at 1 mg/kg did not prevent MDA responses at all. Similarly, focusing on MDA parameters, WIN at the effective dose of 10 mg/kg showed a marked action in decreasing ictal events severity from 20^{th} min of observation on, with respect to WIN at lower doses that do not significantly modify electric activity. As a matter of fact, CB_1 receptors located in the pre-synaptic terminals of central synapses stabilize membrane potentials by adjusting ion currents and inhibit the release of neurotransmitters (Pan et al., 1996; Deadwyler et al., 1995). Accordingly, cannabinoids participate to short- and long- term synaptic plasticity including: depolarization-induced suppression of excitation (DSE) and inhibition (DSI) by respectively reducing glutamate or GABA, or long-term depression of excitatory and inhibitory signals (Ligresti et al., 20161; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). In detail, CB_1-mediated DSE has been hypothesized to be involved in the reduction of the seizure discharge in hippocampal cultures (Deshpande et al., 2007). A prominent line of research tend to support that the antiepileptic properties of CB agonists may be due to a modulation mainly directed towards the inhibition of the glutamatergic

neurotransmission rather than GABA release (Monory et al., 2006). Indeed, the importance of cannabinoid modulation of excitatory glutamatergic transmission is strongly suggested by the widely reported presence of CB_1R in glutamatergic terminals whose altered expression is associated to an unbalance of neuronal excitability driving to pro-epileptic states (Ludànyi et al., 2008; Monory et al., 2006; Lutz, 2004). Though, in the MDA experimental rat model of focal epilepsy, the lack of a complete blockade of WIN-induced effect after pre-treatment with a selective CB_1 antagonist, AM251, (Rizzo et al., 2009) raised the possibility of further mechanisms for CB-mediated modulation of hippocampal seizures. In this regard, several evidence report a wide distribution of CB_2R in neuronal and glial cells in different CNS areas such as cerebral cortex, hippocampus, thalamus, brain stem and cerebellum (Gong et al., 2006), suggesting a potential implication of CB_2 receptors, besides CB_1R role, in mediating CB signalling (García-Gutiérrez et al., 2012). To complete the pharmacological characterization of WIN on CB receptors, it was administered the antagonist/inverse agonist, AM630, known for its high potency and affinity for rat CB_2 receptors (Bolognini et al., 2012; Mukherjee et al., 2004). These results showed that, when compared to the effect induced by WIN alone, the co-treatment with AM630 and WIN significantly reduced the severity of ictal events and, even more, the percentage of responses to the stimulation, suggesting that AM630 improves WIN efficacy. A possible reduced proneness to the epileptogenic phenomena, as revealed by the increase of onset time, is not associated to significant differences in MDA and AD parameters between the AM630-WIN and WIN groups. Taken together, these data might suggest that the efficacy of the co-treatment is exerted mainly by augmenting seizure threshold in the DG, rather than on the epileptic discharge, once elicited. On the basis of the enhancement of WIN-induced effects following AM630 pretreatment, one group of animals was treated with the CB_2 antagonist/inverse agonist to assess its efficacy when administered alone, but this treatment did not alter the characteristics of epileptic discharge, suggesting that

CB_2 exerts no direct effects on hippocampal hyperexcitability in MDA model. From a pharmacodynamic point of view, it can be hypothesized that WIN may have greater occupancy at CB_1R when CB_2 receptors are antagonized by AM630, hence eliciting a better response by facilitating WIN selectivity on CB_1-mediated pathway. Considering that the present study suggest the endogenous CB_1R, rather than CB_2, to be majorly implicated in WIN modulation of hyperexcitability, it is of interest to unveil the downstream pathway involved in cannabinoid complex antiepileptic mechanisms, apart from the well-known CB_1-dependent retrograde signalling (Payandemehr et al., 2015; Hill et al., 2013; Jones et al., 2012). In this regard, the eCB system and NO/cGMP signalling were found to be mutually involved in several processes in the CNS (Azad et al., 2001; Ghasemi et al., 2007; Howlett et al., 2004; Stefano et al., 1998).

Putative implication of NO/cGMP signalling in CB effects in the MDA model

In line with this, the subsequent objective that I pointed to follow was the influence of nitrergic system in cannabinoid effects within the context of hyperexcitability. Authors have reported that in the brain excitability and synaptic function may be oppositely modulated by either endogenous CB signalling or NO-dependent cGMP pathway, for instance through the control on neurotransmitter release and ion channels conductance (Castillo et al., 2012; Ahern et al., 2002; Robello et al., 1996). To this point, our laboratory previously yielded evidence of the importance of nNOS/sGC on MDA phenomena (Sardo et al., 2006) and on the modulation of therapeutic potential of anti-epileptic drugs (Ferraro and Sardo, 2009; Sardo et al., 2009; 2008). The usage of different drugs active on nitrergic pathway is aimed to individuate eventual points of interaction with cannabinoid system. Initially, it was tested 7NI, a preferential nNOS inhibitor, blocking NO synthesis, that was reported to modulate epileptic phenomena with a neuroprotective effect (Banach et al., 2011; Sardo et al., 2006; Borowicz et

al., 2000). In a dose-response assessment in the MDA model, 7NI distinctly reduced the number of MDA responses to the AB stimulation only when administered at 50 mg/kg; whereas 7NI at 35 and 20 mg/kg showed weaker effects. As well, 7NI significantly reduced the durations of MDA and AD parameters only after treatment at 50 mg/kg, also when compared with lower doses. This is in line with what previously found about treatments reducing NO levels in the brain, but also drugs as ODQ, impairing NO-dependent cGMP-activated pathway, via the blockade of sGC (Banach et al., 2011; Sardo et al., 2006).

After this step, aiming to test if a combination of drugs could improve single treatment efficacy, doses that individually resulted non-effective were co-administered, so that the possible appearance of an effect would be attributable to their simultaneous action. To this purpose, in the MDA model animals were pre-treated with 7NI at 20 mg/kg before WIN at 5 mg/kg. My data recorded a noticeable boost of protection in 7NI pre-treated animals, highlighting a potentiation of antiepileptic outcomes within the presumed time frame of WIN efficacy. As a matter of fact, the scarce effect on the percentage of protection displayed by single injection of WIN at 5 mg/kg became significant, in the same lapse of time, when 7NI comes into play. Similarly, MDA parameters were significantly altered in 7NI-WIN group with a dampened susceptibility to epileptic discharge and a reduced intensity of paroxysmal activity once elicited, within the timeframe of WIN efficacy starting from the first 10^{th} min. All together, these data may hint that the interplay managed to control the typical manifestations of the MDA. However, it is conceivable that the co-treatment reduced the excitability of DG neurons, preventing their proneness to altered discharge, via a probable augmentation of the activation threshold in the context of the glutamatergic hippocampal–parahippocampal circuitry. Effects of 7NI–WIN co-administration were counteracted pre-treating animals with AM251; indeed, in this group the protection against MDA disappeared and MDA parameters were not significantly modified compared to controls. Hence, this

outlines the influence of CB_1 receptors in the control of MDA-induced epileptogenesis and that a linkage in the communication between CB and NO systems is represented by CB_1R-activated mechanisms. In this context, to further explore the contribution of NO production in CB effects, I administered arginine, the precursor of the synthesis of NO from nNOS, that previous reports individuated as a pro-convulsant that increases levels of nNOS in epileptic models (Sardo et al., 2006; Bhardwaj et al., 1997). When ARG is co-administered with WIN at maximal dose, protective effects of WIN are reduced, especially regarding severity of paroxysmal discharge. Indeed, MDA parameters did not appear to be modified versus baseline, though a non-significant reduction of MDA responses is found in the early stimulations. These opposed outcomes confirmed the specific implication of nitric oxide production in WIN-mediated effects.

Since NO primary target is cGMP-activated signalling cascades, it would be a point of interest to assess if NO downstream effector is implicated in cannabinoid neuromodulation. Indeed, the administration of ODQ alone, a specific inhibitor of the sGC, was previously discovered by Sardo et al., 2006 to induce a significant decrease of the severity of ictal events, but no change in the MDA responses, in any case suggesting a functional involvement of the NO/sGC metabolic pathway in the DG paroxysmal activity. In this light, in the present research, animals were co-treated with WIN and ODQ. The percentage of responses impressively fell when compared to WIN alone, thus suggesting the presence of a stronger control of the excitability of DG neurons by concomitant sGC inhibition and CB activation. The current electrophysiological data providing evidence to the hypothesis of NO-CB co-role, support other findings strongly bridging NO and eCB inasmuch as regulating neuronal hyperexcitability in pathophysiological states (Bahremand et al., 2009; Jones et al., 2008; Makara et al., 2007; Stringer and Erden, 1995). Nevertheless, studies about the pathophysiology of epileptic phenomena may produce conflicting results, (Löscher, 2011;

Löscher et al., 1991), therefore the promising treatment with the subeffective doses of NO-active and CB drugs were tested in pilocarpine acute model of TLE in order to provide a further behavioural characterization.

Involvement of NO/cGMP signalling in CB effects in the pilocarpine model

In the pilocarpine model, WIN and 7NI were initially administered alone to assess their impact on the behavioural manifestations of acute epileptic phenomena. The onset of pilocarpine maximal effect, as reported in the literature (Curia et al., 2008), is recorded around half an hour after pilocarpine injection, reaching the most severe scores, but symptoms lasts for hours. When individually administered, the effective doses of WIN (10 and 5 mg/kg) and 7NI (50 and 35 mg/kg) not only averted score-5 seizures within the two-hour observations, but also reduced related mortality, according to a dose-dependent pattern. The high mortality rate here observed in control rats is consistent with the data on mortality of rats after 24 hours pilocarpine SE reported by other groups (Jones et al., 2012; Cuellar-Herrera et al., 2010).

My study has shown that a single administration of WIN at maximal dose dramatically boosts survival up to 100%. This favourable effect of WIN on post-SE mortality could be also due to its anti-inflammatory, hypothermic and anti-oxidative effects (Suleymanova et al., 2016; Ma et al., 2014; Marchalant et al., 2007). Moreover, animals treated with WIN or 7NI at the effective doses displayed the less severe signs of pilocarpine scoring scale along the observation period. Particularly, the effect of WIN at 10 mg/kg started immediately, attenuated the intensity of pilocarpine-induced seizures from 30 min and averted behavioural symptoms from 90 to 120 min of the experimental session; whereas, the administration of 7NI at both 50 and 35 mg/kg was effective in thwarting the intensity of seizure scores within the observation, though only in the last 70 minutes, without influencing latency. In support of a

more complete action of WIN, latencies to the first seizure state were also affected in WIN at 10 mg/kg. WIN at 5 mg/kg had a similar ongoing, though it did not properly protect animals within the pilocarpine maximal effect. WIN activity was displayed to be dependent on CB_1 receptors activation in that the CB_1 antagonist, AM251, counteracts WIN effective dose and this represents a first specific outcome about CB_1R role in the acute pilocarpine model. In analogy with the MDA experimental procedures, having found WIN at 1 mg/kg and 7NI 20 mg/kg as ineffective doses for the parameters considered in this model, a parallel co-treatment study was conducted to explore behavioural outcomes, as well. The injection of WIN and 7NI protected animals from severe seizures and mortality within 24 h, similarly to the individual effective doses of WIN and 7NI. Noticeably too, the co-treatment emerged as fruitful in delaying the latency of pilocarpine-induced onset and attenuating the intensity of behavioural symptoms within the experimental period, without the side effects observed here for the higher doses of cannabinoids and reported previously (Wallace et al., 2003; 2001). Otherwise, pretreatment with AM251 reverts 7NI–WIN protection from the most severe seizures and, consistently, reduced latency to first symptoms and increased mortality versus 7NI-WIN alone, thereby highlighting the importance of CB_1R not only in the activity of WIN alone but also in the 7NI potentiation of WIN effects.

Since behavioural results supply a novel behavioural characterization of the drugs used, it is worth speculating on the probable synaptic processes involved. In the acute pilocarpine model, the chemo-convulsant acts on the muscarinic acetylcholine receptor (mAChr) M1 subtype, responsible for seizure initiation, and consequently triggers NMDA signalling, implicated in seizure maintenance (Curia et al., 2008 ; Hamilton et al., 1997; Clifford et al., 1987). Interestingly enough, WIN, but not 7NI, was able to delay the onset of first seizure signs, hinting the idea that only CB agonism could counterbalance muscarinic epileptogenesis. A neuroprotective production of endocannabinoids following mAChr

activation in mouse pilocarpine model was indeed found (Kow et al., 2014); thus, conceivably, NO signalling is not involved in seizure initiation. Furthermore, treatments with WIN or 7NI attenuate the intensity of behavioural signs, due to the possible mitigation of NMDA-mediated seizure maintenance (Kow et al., 2014). In this regard, these data support the idea that both cannabinoid and nitrergic systems act on NMDA excitatory pathways, maybe through independent mechanisms (Howlett et al., 2010; Fagni and Bockaert, 1996), and this common synaptic target may be responsible for their individual and synergic antiepileptic activity. Notably, the co-treatment may influence both mAChr-induced initiation and NMDA-sustained maintenance of seizures in this model, in line with the action of WIN alone when effective, hence leaning toward a possible facilitation of CB-mediated pathway following nNOS blockade. Moreover, it is arguable that a possible involvement of NO is to be considered downstream CB activation, especially for muscarinic phase. This is in agreement with data here obtained from MDA model since, by modulating nNOS and sGC activity via diverse drugs, nitrergic pathway could be hypothesized to be targeted as a neuromodulator after previous CB_1 activation (e.g. under WIN application).

It can be asserted that nNOS inhibition in particular promotes a potentiation of CB effects, since I observed that antiepileptic actions were boosted within an early time frame that in the initial dose-dependent studies in the two models was pointed out as mainly subjected to WIN action, consistently with other reports both on WIN and 7NI dynamics (Rizzo et al., 2009;Valiveti et al., 2007; Kalisch et al., 1996). At molecular level, a recent perspective implies CB receptors and NO also via the co-internalization of CB_1R and NR1 subunit of NMDA in order to offset against NO production, with the ultimate goal of overriding CB protection from brain damage (Sánchez-Blázquez et al., 2013). My results, obtained through CB_1R inhibition before 7NI–WIN co-treatment, well fit within this context and show that cooperation between the effects of 7NI and WIN stops when CB_1 activation is cut out. On this

point, though my experimental approach does not allow us to discriminate whether the cooperative action of WIN and 7NI is due to linked or independent networks, several evidence point to a control of CB_1R pathway on the production of NO by NOS isoforms. Indeed, in mice neuron cultures it was observed that the inhibition of NO production by WIN is reversed in CB_1R-knockouts, along with an increased basal activity of nNOS (Kim et al., 2006 b). It was suggested that CB_1R exerts the abovementioned effect on NO production decreasing the calcium influx and therefore the activity of Ca-dependent nNOS isoform (Hillard et al., 1999). In this view, cannabinoid ability to reduce the release of glutamate from synaptic terminals could impair NMDA-dependent calcium signalling, thus lowering post-synaptic Ca-mediated NO production and ultimately impairing cGMP formation (Fig. 22).

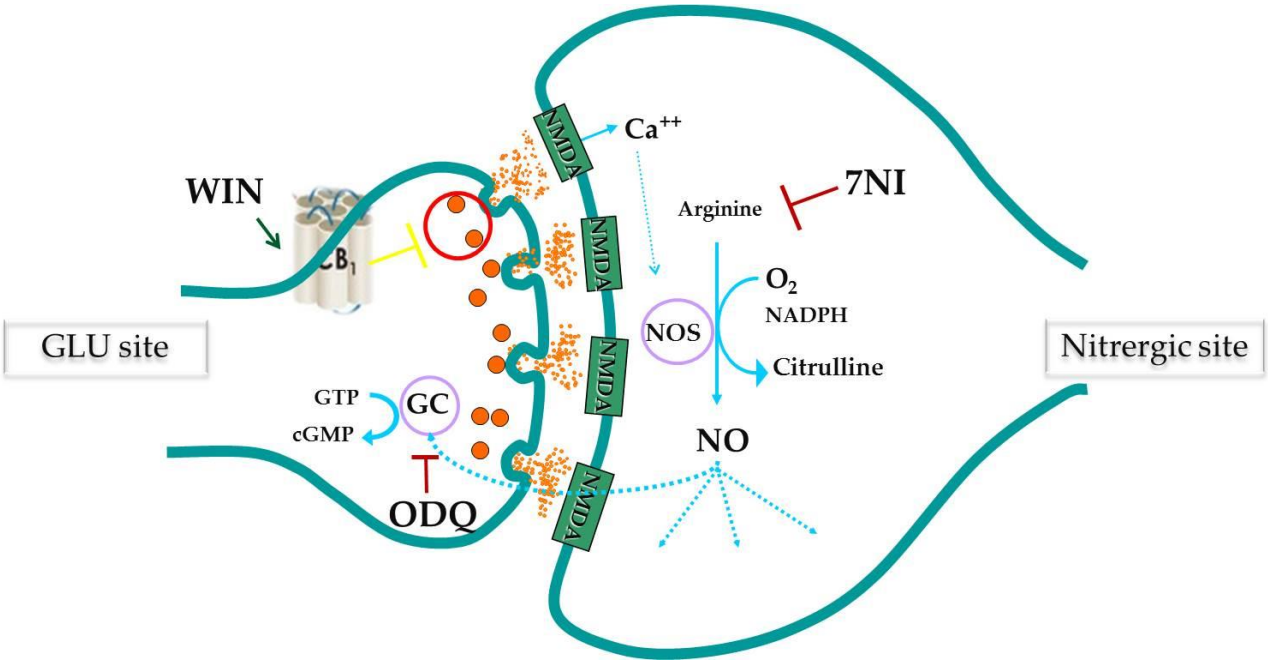

Fig. 22. Representation of the molecular players targeted by WIN, 7NI and ODQ. Considering pre-synaptic CB_1 localization in glutamatergic (GLU) terminals, WIN could reduce GLU release, lowering post-synaptic intracellular Ca^{2+} that is essential for NO production and consequent activation of pre-synaptic sGC.

Taken together, the co-treatment results, applying 7NI, ODQ and ARG to cannabinoid-activated cells, added knowledge to the formerly suggested interplay between cannabinoid and nitrergic systems in the context of synaptic transmission (Hillard et al., 1999), particularly regarding the mechanisms underlying neuronal excitotoxicity (Kim et al., 2006 a). Indeed, my study seem to confirm a functional antagonism between CB receptors and NO, since the hindrance of NO signalling favours CB-mediated antiepileptic actions, though on this issue there are still no concerted opinions in literature. In accordance with these findings, growing pieces of evidence prompted to suggest that blocking nNOS via 7NI treatment protects against hyperexcitability phenomena (Banach et al., 2011; Takei et al., 2001) and other neurodegenerative processes (Yuste et al., 2012; Bostanci and Bagirici 2007; Hantraye et al., 1996). Noteworthy, albeit main analogies, some variances came to light as concerns the protective doses in the two models, putatively due to the different mechanisms behind epileptic phenomena, and this led to the choice of distinct WIN doses for the co-treatments. On the other hand, opposite results on the influence of 7NI on CB agonists were reported in another model of epilepsy in mice (Bahremand et al., 2009), in agreement with other authors claiming the pro-convulsant activity of NOS inhibitors (de Vasconcelos et al., 2000; Del-Bel et al., 1997; Przegalinski et al., 1996). The difficulty of fully understanding the function of nitrergic system in epileptic studies is prominent (Banach et al., 2011), since discrepancies arise even using the same protocol (Payandemehr et al., 2014; Bahremand et al., 2009; Van Leeuwen et al., 1995), but especially with differential paradigms, species and treatments (Löscher et al., 1991). This validates the effort to use a dual experimental approach to provide a wider perspective of the CB–NO cross-talk in the epileptogenic phenomena. Lastly, the CB–NO possible interaction here assessed in the context of the modulation of paroxysmal events, may have a broader connotation. Indeed, these systems mutually exert a refined control of synaptic transmission with a relapse on multiple processes in various brain areas

(Kim et al., 2006a, b; Hillard et al., 1999; Waksman et al., 1999), postulating nNOS activity as modulated by cannabinoid effects.

All this evidence support the idea that the activation of CB_1-pathway could have among its downstream effects the blockade of post-synaptic NO production, directly targeting nNOS or more complex cellular mechanisms.

Further factors involved in CB signalling in electrically-induced seizures

Among CB-mediated complex cellular mechanisms, the subsequent aim of this study endorsed recent trends on the relevance of cannabinoid post-synaptic signalling, directed specifically towards CB_1 receptors or CB_1-activated downstream processes. Indeed, TRPV1 has emerged as a mediator of CB post-synaptic cascades since vanilloids were found to be implicated in the fine tuning of CB-induced regulation of synaptic strength in various processes (Di Marzo and De Petrocellis, 2012), particularly in different experimental models of epilepsy (Jia et al., 2015; Naderi et al., 2015; Gonzalez-Reyes et al., 2013; Manna and Umathe, 2012; Fu et al., 2009). To serve the purpose of assessing the possible role of TRPV1 in cannabinoid-induced antiepileptic effect, I pharmacologically manipulated with TRPV1-active drugs the pathway triggered by WIN, that has been reported to exhibit a variegated activity on TRP channels (De Petrocellis and Di Marzo, 2010; Qin et al., 2008).

At first, the single involvement of TRPV1 in the modulation of MDA-related electrophysiological phenomena was analysed. Animals were treated with different doses of the couple of TRPV1 agonist and antagonist mostly used in this field: capsaicin and capsazepine. The highest dose of CAP produced an intensification of the epileptic discharge characterized by related changes in the MDA parameters, i.e., reduction of time of onset and elongation of MDA and AD durations. What was here found is in line with other reports describing CAP promoting firing rate in epileptic hippocampal slices (Saffarzadeh et al.,

2015; Kawahara et al., 2011; Bhaskaran and Smith, 2010) and showing pro-convulsant activity in behavioural models (Jia et al., 2015). A similar dose-response evaluation was conducted for CPZ-treated groups. The only dose that resulted effective was the highest one (2 mg/kg). In this case, the effects were opposite to CAP: increase of the time of onset and shortening of MDA and AD durations, therefore showing an antiepileptic outcome and suggesting that in a hyperexcited state TRPV1 channels could be tonically active. Never did CPZ arrest MDA response after AB stimulation. In the light of this initial evidence, a modulation by TRPV1 of paroxysmal MDA events, quantitatively and qualitatively distinct from WIN antiepileptic effects, emerged.

Taken this into account, WIN effects were subsequently evaluated co-administering animals with CAP or CPZ. In CAP–WIN group, the % of protection from MDA was reduced with respect to single WIN action at 10 mg/kg, also limiting the interval in which WIN is able to block MDA response. Conversely, to antagonize TRPV1 function in the timeframe of CB1 antiepileptic action, CPZ at 2 mg/kg was administered before the effective dose of WIN. It resulted that WIN and CPZ action was potentiated with respect to WIN at 10 mg/kg, especially in the % protection from MDA response, since the number of animals not responding to stimulation is markedly augmented. The lack of a correlated statistical power of changes in MDA parameters is probably due to the dampened number of animals responding to stimulation. Changes in the % of responding animals would indicate an involvement of TRPV1 on CB_1R control of the susceptibility of DG neurons when electrically triggered, i.e., whether epileptic discharge starts or not. Considering CPZ–WIN ability to enhance antiepileptic outcomes, the influence of CPZ on the sub-effective dose of WIN at 5 mg/kg was then assessed. Results showed that % of protection from MDA was not altered after pre-treatment with CPZ at 2 mg/kg, albeit the duration of MDA and AD parameters was statistically reduced when compared to group treated with only WIN at 5 mg/kg; hence this

combination likely managed to turn into effective to some extent the subeffective dose of WIN. Noteworthy, neither WIN 5 mg/kg alone was ever able to stop MDA response nor the co-treatment with CPZ influence the subeffective dose of WIN in the blockade of the paroxysmal discharge.

This evidence shed light on the effects of capsaicin and capsazepine that in the recent years have been increasingly studied in different models of epilepsy, putatively modifying neuronal firing with opposite outcomes (Jia et al., 2015; Iannotti et al., 2014; Gonzalez-Reyes et al., 2013; Manna and Umathe, 2012; Bhaskaran and Smith, 2010). It is established that the cation-channel TRPV1, when activated, induces neuronal depolarization and contributes to generate action potentials that ultimately facilitate the release of glutamate (Starowicz et al., 2007; 2008; Xing and Li, 2007). Conversely, cannabinoid signalling has been recognized to act pre-synaptically by decreasing intracellular calcium overload and reducing neurotransmitters release (Freund et al., 2003; Alger, 2002), but also to trigger complex inhibitory pathways in the post-synaptic site, contributing to reduce excitability level such as the modulation of TRPV1 and nitrergic system (Castillo et al., 2012; Wang et al., 2012; Hong et al., 2009; fig. 23 a and b).

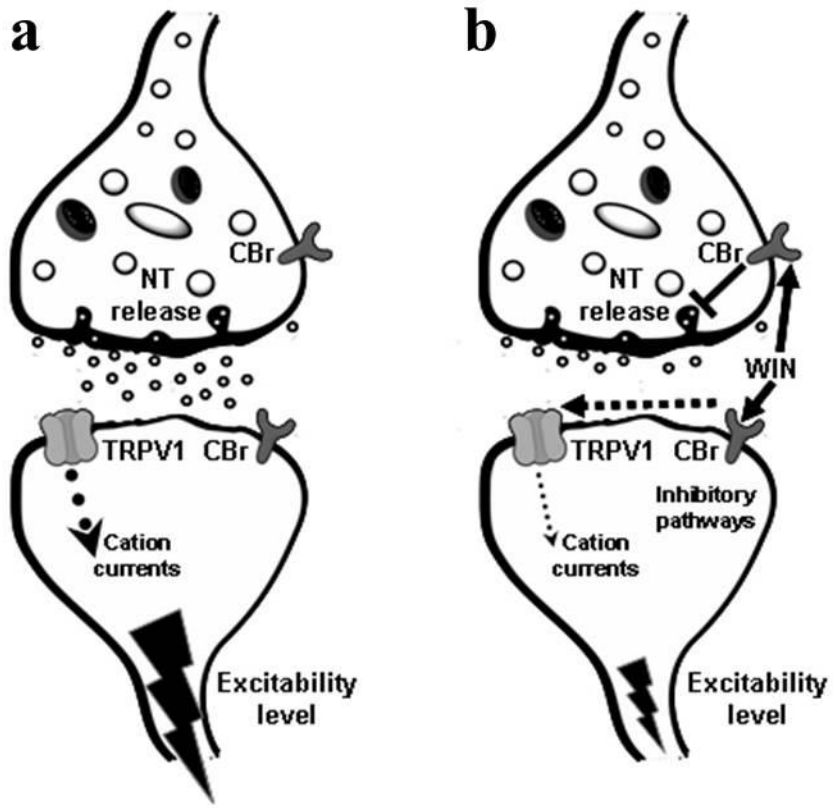

Fig. 23. *a, b.* **Proposed mechanisms for CB-TRPV1 activity in the MDA**. Neurotransmitter (NT) release is modulated by pre-synaptic CBR activation. The different excitability levels of DG neurons could be influenced by WIN administration acting on: post-synaptic CBR eliciting inhibitory pathways; TRPV1, responsible for cation currents or a possible impairment of CB system on TRPV1 function. Continue arrows (→) indicate the direct activation of receptors; the barred lines (⊣) stand for antagonism or blockade of a process; dashed arrows (- - →) show a modulatory action and spotted arrows of different thickness (••••➔) indicate TRPV1-cation currents of different intensity.

Yang et al. hypothesized that WIN action is utter enough when exerted on CB_1 and TRPV1 co-localized at the post-synaptic terminal (2013). As a result, these receptors are subjected to a protein–protein interaction that can decline depolarizing currents, lastly suppressing TRPV1-linked downstream events. This reinforces the direct evidence that coincident CB_1 activation and TRPV1 antagonism facilitate antiepileptic outcomes, as well as TRPV1 agonism interferes with CB_1-mediated neuroprotection in hyperexcitability. A schematic representation of the possible sites of interplay of these systems is reported in fig. 23 c and d.

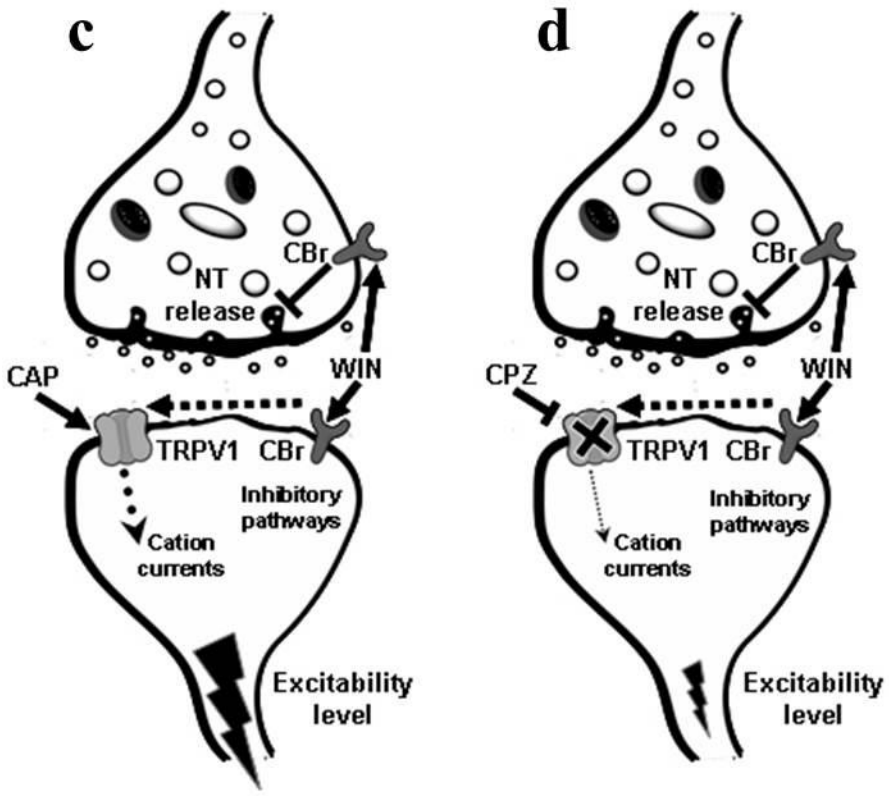

Fig. 23. *c, d.* **Proposed mechanisms for CB-TRPV1 activity in the MDA**. Capsaicin (CAP) and capsazepine (CPZ) oppositely gate post-synaptic TRPV1, hence modulating WIN effect on excitability levels. Continue arrows (⟶) indicate the direct activation of receptors; the barred lines (⟙) stand for antagonism or blockade of a process; dashed arrows (- - →) show a modulatory action and spotted arrows of different thickness (••••➤) indicate TRPV1-cation currents of different intensity.

Suggested points of interaction between cannabinoid and nitrergic systems in hyperexcitability

A novel interpretation could be suggested considering the implication of post-synaptic NO in CB_1-TRPV1 signalling that has been recently proposed in various neuronal processes (Batista et al., 2015; Aguiar et al., 2014; Zschenderlein et al., 2011). Indeed, TRPV1 were found to target post-synaptic nNOS, since systemic administration of CAP increased NO synthesis in the hypothalamus and amygdala in rats, whereas TRPV1 antagonists revert the activity of NO

donors (Lisboa et al., 2013; Okere et al., 2000). Noteworthy, a relevant paper stated that long-term potentiation in the lateral amygdala is dependent on TRPV1, gated by CAP and modulated by cannabinoids, with a relapse on NO-mediated glutamatergic transmission (Zschenderlein et al., 2011). These authors revealed that "anandamide modulates NO levels by two independent pathways: (1) diminishing the NOS activity via cannabinoids; and (2) stimulating NO synthesis via TRPV1". Similarly, the activity of cannabinoids on behavioural responses was correlated to NO levels via modulation of TRPV1 (Batista et al., 2015).

In this light, it could be hypothesized that in the hippocampal paroxysmal discharge TRPV1 could affect the nitrergic modulation of the antiepileptic effects exerted by WIN. Thus, some pilot experiments were conducted co-administering 7NI or ARG before CAP and WIN in the MDA, in order to test if NO levels could alter capsaicin impairment of WIN effects. My results proved in both groups (7NI-CAP-WIN and ARG-CAP-WIN) the persistence of the antiepileptic effect of WIN, though its efficacy in presence of CAP was related to the nNOS activity. Indeed, considering MDA parameters, 7NI-CAP-WIN revealed a marked anti-epileptic effect with respect to ARG-CAP-WIN and, as for the % of responses, 7NI was found to attenuate CAP impairment of WIN protective effects since animals did not respond to stimulation for a more extended period not only comparing with % of responses of ARG-CAP-WIN group, but also with CAP-WIN treatment alone. Overall, I observed that the blockade or the promotion of the NO production modified oppositely the influence of CAP on WIN antiepileptic effects. These preliminary findings could corroborate the importance of NO in the intracellular action of TRPV1 and CB_1 receptors. Noticeably, this modulatory mechanism may occur by an independent action of the two receptors converging on the same target or, alternatively, TRPV1 could represent a link between CB_1 and nNOS functions. In particular, WIN effects on NO production may be exerted by the direct modulation of post-synaptic TRPV1.

CONCLUSIVE REMARKS AND FUTURE PERSPECTIVES

The present research yielded knowledge on novel CB_1-dependent interactions in the modulation of neuronal function in the hippocampus within the remit of hyperexcitability phenomena.

The current findings support the speculation that a pathophysiological unbalance of CB_1/TRPV1 and nitrergic signalling systems could be associated with bioelectrical alterations of synaptic processes, thus influencing experimental epileptic conditions. Cannabinoids and related pharmacological tools stand out for their therapeutic implications in refractory, focal epilepsy that frequently fail to be controlled, though backward opinions claim that cannabinoid psychoactive toxicity make their use impractical. Remarkably, in this study, the pharmacological manipulation of CB pathway through nitrergic signalling was a promising strategy to finely tune WIN efficacy in two parallel models of temporal lobe epilepsy in the rat so as to abolish seizures, but to be devoid of adverse consequences. Nonetheless, further studies on epilepsy models are necessary to deepen understanding of mechanisms involved. The development of novel therapeutic agents enhancing anticonvulsant action of cannabinoids without unwanted side effects can establish a solid rationale for additional research to act on intractable seizures. Hence, pre-treatment with drugs acting on nitrergic system may have face validity within the clinical setting and this fosters the urgency to further explore the pharmacological potential properties of cannabinoids.

The results of this research have been included in the following recent publications:

-"Carletti F, Gambino G, Rizzo V, Ferraro G, Sardo P. 2016. *Epilepsy Research* 122:56–65"

-"Carletti F, Gambino G, Rizzo V, Ferraro G, Sardo P. 2015. *Neuroscience* 303:149–159"

-"Rizzo V, Carletti F, Gambino G, Schiera G, Cannizzaro C, Ferraro G, Sardo P. 2014. *Epilepsy Research* 108 (10):1711–1718".

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