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EFFECTS OF CANNABIDIOL AND CANNABIS EXTRACTS IN MODELS OF CONVULSION AND EXCITOTOXICITY

TESI DI DOTTORATO

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Nothing in this world is to be feared... only understood.
Marie Curie

Science is a wonderful thing if one does not have to earn one's living at it.
Albert Einstein

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INTRODUCTION

Cannabis sativa L.

Cannabis sativa is an herb that has been used by humans for millenniums for its fibre, known as hemp, for its psychological and physiological effects and for the nourishment of its oil-bearing seeds. The fibre of the plant, cultivated as hemp, has numerous textile uses. Its seed is a valuable source of protein and unsaturated oils. The flowers (and to a lesser extent the leaves and stems) contain psychoactive and physiologically active chemical compounds known as cannabinoids that are consumed for recreational, medicinal, and spiritual purposes. When so used, preparations of flowers (marijuana) and leaves and preparations derived from resinous extract (hashish) are consumed by smoking, vaporizing and oral ingestion. The major biologically active chemical compound in Cannabis is Δ-9-tetrahydrocannabinol, commonly referred to as THC. The major difference between the variety grown for industrial use and the one primarily used for production of recreational and medicinal drugs, is the amount of Δ-9-tetrahydrocannabinol (THC) secreted in a resinous mixture by epidermal hairs called glandular trichomes.

Ingestion of Cannabis sativa preparations results in an intoxication characterized by sedation, cognitive dysfunction, failure to consolidate short-term memory, alteration in time assessment, perceptual changes, motor in-coordination and poor executive function (reviewed in Abood and Martin, 1992; Dewey, 1986; Hollister, 1986; Pertwee, 1988). Most of these effects are due to the action of THC, the main psychoactive substance found in the Cannabis plant. THC was isolated by Raphael Mechoulam and co-workers in 1964, and its mechanism of action was disclosed in 1990, when the first cannabinoid receptor (CB_1) was cloned (Matsuda et al., 1990) In fact many of the pharmacological actions of THC result from its binding to the cannabinoid receptor CB_1. The four symptoms that are often used to define cannabinoid intoxication in the rodent - hypothermia, rigid immobility,

analgesia and decreased motor activity (Adams and Martin, 1996) - are absent in mice in which the cb1 gene has been deleted by targeted recombination (Ledent et al., 1999; Zimmer et al., 1999). Cannabinoids are the most distinctive and specific class of compounds known to exist only in the cannabis plant. They are a group of terpenophenolic compounds, with very low solubility in water, but good solubility in most organic solvents. Other natural cannabinoids are cannabinol (CBN), cannabidiol (CBD), one of the most promising cannabinoid the researchers are investigating on, cannabigerol (CBG), cannabichromene (CBC), and delta-8-tetrahydrocannabinol (Δ-8-THC).

The endocannabinoid system

The endocannabinoid system is made up of two cannabinoid receptors, called CB_1 and CB_2, their endogenous ligands, called the endocannabinoids, the proteins for their synthesis and inactivation, and other molecular targets for the endocannabinoids. The endocannabinoids known by far are anandamide (N-arachidonoyl-ethanolamine, AEA) and 2-arachidonoyl-glycerol (2-AG) discovered in the 1990s, whilst 2-arachidonyl-glyceryl ether (noladin, 2-AGE), O-arachidonoyl-ethanolamine (virhodamine) and N-arachidonoyl-dopamine (NADA) have been proposed as cannabinoid receptor agonists in this decade (De Petrocellis et al., 2004).

Endocannabinoids release from neurons and their deactivation

Endocannabinoids synthesis is stimulated by intracellular Ca^{2+} elevation (Bisogno et al., 1997; Cadas et al., 1996; Di Marzo et al., 1994). Anandamide and 2-AG are hydrophobic compounds, so they tend to remain associated with lipid membranes where they are produced. They can approach the CB_1 receptors by lateral membrane diffusion, but it is likely that some extracellular lipid-binding proteins help to deliver endocannabinoids to their cellular targets (Piomelli, 2003). Reuptake of endocannabinoids by a yet

uncharacterized carrier and intracellular hydrolysis are the two mechanisms, which cooperate in attenuating endocannabinoid signalling in the brain (Piomelli, 2003). A series of pharmacological transport inhibitors have been synthesized; these molecules - the prototype of which is AM404 - (Beltramo et al., 1997; Beltramo et al., 2000; Piomelli et al., 1999), helped to unmask important roles of the endocannabinoid system in the regulation of neurotransmission and synaptic plasticity. Fatty acid amide hydrolase, FAAH, is an intracellular membrane-bound serine hydrolase that breaks down anandamide into arachidonic acid and ethanolamine (Cravatt et al., 1996; Schmid et al., 1985). FAAH is widely distributed in the rat brain, where it is expressed at high concentrations in cell bodies and dendrites of principal neurons (Egertova et al., 2003; Tsou et al., 1998). Monoacylglycerol lipase (MGL), which is responsible for the hydrolysis of 2-AG, has a broad distribution in the central nervous system, which partially overlaps with that of FAAH; however, whereas FAAH is predominantly found in postsynaptic structures, MGL might be mostly associated with nerve endings (Dihn et al., 2002).

CB_1 receptors pharmacology in the CNS

The cannabinoid receptor type 1, CB_1, is a G protein-coupled receptor that is found in the brain and is activated by THC, anandamide and 2-AG (Pazos 2005). The receptor is coupled to a $G_{i/o}$ protein, and upon its stimulation several intracellular signal transduction pathways are activated: inhibition of the enzyme adenylate cyclase, inhibition of N- and P/Q-type calcium channels, activation of inwardly rectifying potassium channels, and activation of mitogen-activated protein kinases (Fowler, 2003). CB_1 receptors are thought to be the most widely expressed G protein-coupled receptors in the brain. Varying levels of CB_1 expression can be detected in the olfactory bulb, cortical regions (neocortex, pyriform cortex, hippocampus, and amygdala), several parts of basal ganglia, thalamic and hypothalamic nuclei and other

subcortical regions, cerebellar cortex, and brainstem nuclei (e.g. the periaqueductal gray) (Howlett et al., 2002). In these structures CB_1 receptors demonstrate a presynaptic location, and this characteristic suggests that the endocannabinoid system could play an important role in synaptic neurotransmission (so-called "endocannabinoid retrograde signalling"). The hippocampus has an essential role in the formation of new memories, and endocannabinoids play an important role in the modulation of memory in this area of the brain (Robinson et al., 2004). Depolarization-induced suppression of inhibition (DSI), is a very common form of short-term plasticity in which the depolarization of a pyramidal neuron in the CA1 field of the hippocampus, induces a reduction in GABA inhibitory inputs. DSI is mediated by endocannabinoids (Alger, 2002; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Yoshida et al., 2002), and has been shown to occur in other areas of the brain (i.e. cerebellum) (Kreitzer and Regehr, 2001; Trettel and Levine, 2003). Cannabinoid agonists seem to have an influence on affective states (Viveros et al., 2005), and inactivation of CB_1 receptors causes anxiety-like and aggressive responses in rodents (Martin et al., 2002; Navarro et al., 1997). Cannabinoids might contribute to the regulation of the basal ganglia function (Fernández-Ruiz and Gonzáles, 2005), and may be involved in the modulation of motor activity (Giuffrida et al., 1999; Kettunen et al., 2005; Kyriakatos and Manira, 2007). Beside their actions in the amygdala, which seems to be the site of cannabinoid central analgesia (Katona et al., 2001; Macdonald and Mascagni, 2001; Martin et al., 1999), cannabinoid agonists can influence the central processing of pain in hindbrain (Jennings et al., 2001; Lichtman et al., 1996; Meng et al., 1998), and systemic administration of CB_1 antagonists produces hyperalgesia in rats and mice (Calignano et al., 1998; Richardson et al., 1997; Strangman et al., 1998). Though central and peripheral actions could underlie the analgesic properties of cannabinoid drugs (Cravatt and Lichtman, 2004; Iversen and Chapman, 2002), recent findings seem to indicate the CB_1

receptors expressed on the peripheral axons of primary sensory neurons, as the main target for substantial analgesia for somatic and visceral pain, as well as in inflammatory and neuropathic pain (Agarwal et al., 2007).

Endocannabinoid-mediated retrograde transmission is also involved in the depolarization-induced suppression of excitation (DSE), in principal neurons in hippocampus and cerebellum. Similarly to DSI, DSE is induced by neuronal depolarization, and consists of a transient depression in neurotransmitter release. DSE targets glutamatergic axon terminals, and thus reduces excitatory input to the affected cell (Alger 2002; Ohno-Shosaku, 2002; Kreitzer and Regehr, 2001).

The ability of cannabinoid agonists to inhibit the release of neurotransmitters in the CNS is not restricted to glutamate and GABA. Acetylcholine release is reduced by cannabinoids both in vitro and in vivo, and is enhanced by inactivation of CB_1 receptors (Degroot et al., 2006; Gessa et al., 1998; Gifford and Ashby, 1996; Schlicker and Kathmann, 2001). Since acetylcholine release in the neocortex and hippocampus facilitates learning and memory, its endocannabinoids-mediated decrease might contribute to the negative effects of cannabinoid drugs on cognition.

The endocannabinoid system is also involved in long-term regulation of synaptic plasticity. Long-term depression (LTD), is the weakening of a neuronal synapse that lasts from hours to days, and is induced (in hippocampus) by a persistent weak synaptic stimulation. Evidences that striatal LTD is absent in CB_1-deficient mice and is blocked by the CB_1 antagonist rimonabant suggest that endocannabinoids are involved in this phenomenon; moreover CB_1 agonists can induce LTD (Chevaleyre and Castillo, 2003; Kreitzer and Malenka, 2007; Gerdeman et al., 2002; Ronesi et al., 2004).

The possible postsynaptic location of CB_1 receptors is still under debate. Several reports suggested that these receptors could exhibit a postsynaptic

location (Marsicano et al., 2003; Rodriguez et al., 2001) in rat brain, whereas other works are against these data (Freund et al., 2003).

CB_2 receptors pharmacology in the CNS

CB_2 receptors are mainly expressed in the immune system, and they modulate cytokine release and immune cell migration (Howlett et al., 2002). CB_2 receptors are also expressed on peripheral nerve terminals (Howlett et al., 2002). In the brain they are expressed by microglia and astrocytes, where their role remains unclear (Fernández-Ruiz et al., 2007), on neurons in several brain regions (Onaivi et al., 2006), and in the spinal cord. They have been attributed anti-nociceptive potential (Jhaveri et al., 2007). Activation of CB_2 receptors inhibits adenylyl cyclase (Slipetz et al., 1995) and activates mitogen-activated protein kinase (Bouaboula et al., 1996) through $G_{i/o}$ protein.

Glutamatergic transmission and excitotoxicity

Glutamate and excitatory neurotransmission

Glutamate is the most abundant excitatory neurotransmitter in the mammalian nervous system. It contributes to synaptic neurotransmission, and also to complex physiological processes like memory, learning, plasticity, and neuronal cell death (Dingledine et al., 1999; Ozawa et al., 1998). Glutamate is synthesized in the cytoplasm and stored in synaptic vesicles by the vesicular glutamate transporters (VGLUTs). Following its exocytotic release, glutamate activates ionotropic glutamate receptors for fast excitatory neurotransmission and metabotropic receptors for slower modulatory effects on transmission. To terminate the action of glutamate, Na^+-dependent high affinity glutamate transporters (excitatory amino acid transporters: EAATs) located on the plasma membrane of neurons and glial cells rapidly remove glutamate from the extracellular space (Balcar, 2002; Danbolt, 2001). Most of

the glutamate is released synaptically and transits through the glutamate–glutamine cycle before being stored into synaptic vesicles (Hamberger et al., 1979). Glutamate taken up into glial cells is metabolized to glutamine, which is then transported back into neurons, converted to glutamate and sequestered into synaptic vesicles by the VGLUTs. In brain injury or disease, glutamate transporters can work in reverse and glutamate can accumulate outside cells (Shigeri et al., 2004). Glutamate receptors are transmembrane receptors located on neuron membranes. There are two basic types of glutamate receptor: ionotropic (NMDA receptor, kainate receptor, and AMPA receptor), and metabotropic (mGluR1, mGluR2, mGluR3, mGluR4, mGluR5, mGluR6, mGluR7, mGluR8).

Glutamate ionotropic receptors: NMDA receptors

The NMDA receptor (NMDAR) is an ionotropic receptor for glutamate (N-methyl D-aspartate, NMDA, is the agonist its name refers to). Activation of NMDA receptors results in the opening of an ion channel that is nonselective to cations: it allows flow of Na^+ and small amounts of Ca^{2+} ions into the cell, and K^+ out of the cell. Calcium flux through NMDARs is thought to play a critical role in synaptic plasticity. The NMDA receptor forms a heterodimer between NR1 and NR2 subunits. Multiple receptor isoforms with distinct brain distributions and functional properties have been identified. Each receptor subunit contains two globular structures in the extracellular domain: a modulatory domain and a ligand binding domain. NR1 subunits bind the co-agonist glycine and NR2 subunits bind the neurotransmitter glutamate. The membrane domain consists of three trans-membrane segments and a loop, it is responsible for the receptor's conductance, high-calcium permeability, and voltage-dependent magnesium block. Each subunit has also a cytoplasmic domain, which residues can be modified by protein kinases and protein phosphatases, and can interact with a large number of proteins. Activation of NMDA receptors requires binding of glutamate (or aspartate), and also

requires the binding of the co-agonist glycine. D-serine has also been found to co-agonize the NMDA receptor. In addition, a third requirement is membrane depolarization. A positive change in transmembrane potential will open the ion channel by expelling the Mg^{2+} ion that blocks the channel (Dingledine et al., 1999; Wolosker, 2007).

Glutamate ionotropic receptors: kainate receptors

Kainate receptors, or KARs, are non-NMDA ionotropic receptors, which respond to the neurotransmitter glutamate. They were first identified as a distinct receptor type through their selective activation by the agonist kainate. There are five types of kainate receptor subunits, GluR5, GluR6, GluR7, KA1 and KA2, arranged in different ways to form a tetramer. The ion channel formed by kainate receptors is permeable to Na^+ and K^+ ions. Kainate receptors play a role in both pre- and postsynaptic neurotransmission. They have a somewhat more limited distribution in the brain compared to AMPA and NMDA receptors, and their function is not well defined. They are involved in epilepsy, excitotoxicity, and synaptic transmission of noxious stimuli (Pinheiro and Mulle, 2006).

Glutamate ionotropic receptors: AMPA receptors

The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (also known as AMPA receptor, AMPAR, or quisqualate receptor) is a non-NMDA-type ionotropic transmembrane receptor for glutamate that mediates fast synaptic transmission. Its name is derived from its ability to be activated by the artificial glutamate analog, AMPA. AMPARs are found in many parts of the brain and are the most commonly found receptor in the nervous system. AMPARs are composed of four types of subunits, designated as GluR1, GluR2, GluR3, and GluR4, which combine to form tetramers. Most AMPARs are either homo-tetramers of GluR1 or GluR4, or symmetric 'dimer of dimers' of GluR2 and either GluR1, GluR3 or GluR4. Each AMPAR has four sites to

which a molecule of the agonist can bind, one in each subunit; the channel can open when two or more sites are occupied. AMPARs open and close quickly, and are thus responsible for most of the fast excitatory synaptic transmission in the central nervous system. The permeability of AMPAR to Ca^{2+} and other cations, such as Na^{+} and K^{+}, is governed by the GluR2 subunit (if an AMPAR lacks a GluR2 subunit, then it will be permeable to Na^{+}, K^{+} and Ca^{2+}). The principal ions gated by AMPARs are Na^{+} and K^{+}. The subunit composition of the AMPAR is also important for the way this receptor is modulated. If an AMPAR lacks GluR2 subunits, then it is susceptible to being blocked in a voltage-dependent manner by polyamines, which prevent the flux of K^{+} ions through the channel pore at depolarized membrane potential (Dingledine et al., 1999).

Glutamate metabotropic receptors

Metabotropic glutamate receptors (mGluRs) are members of the G protein-coupled receptor (GPCR) superfamily and they have been shown to play an important role in processes requiring synaptic plasticity, such as learning and memory, neuronal development, and neurodegeneration. mGluRs have been divided into 3 subgroups based on sequence similarities, signal transduction pathways and pharmacology (Conn and Pin, 1997; Dale et al., 2003). They are Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7, and mGluR8). In contrast to ionotropic glutamate receptors, which mediate fast synaptic transmission at glutamatergic synapses, mGluRs often modulate ongoing activity. Postsynaptic mGluRs may modulate membrane properties by second messenger interactions, while presynaptic mGluRs have been shown to control synaptic release; depending on the specific mGluR and its position within brain circuits, the resulting modulation may be facilitatory or inhibitory (Alexander and Godwin, 2006). All mGluRs have seven transmembrane domains, with the intracellular loop between domains 3 and 4

binding a G-protein. Group I mGluRs (mGluR1 and 5) couple to G_q to stimulate phosphoinositide hydrolysis and phospholipase C. Group II (mGluR2 and 3) and Group III mGluRs (mGluR4, 6, 7, and 8) couple to G_i/G_o to inhibit cAMP. In addition to these second messenger pathways, mGluRs can signal by uncoupling of the βγ subunits from the heterotrimeric G protein to directly modulate ion channels. In general, Group I mGluRs are excitatory, acting to enhance neurotransmitter release, potentiate ionotropic glutamate receptors responses and modulate various depolarizing currents. Group II and III mGluRs generally act to reduce neurotransmitter release, and within the axon terminal G-protein effects may include inhibition of high threshold calcium channels, activation of potassium channels and direct inhibition of transmitter release machinery. All mGluRs are expressed on neurons, and mGluR3 and 5 are additionally found on glial cells (De Blasi et al., 2001; Kew and Kemp, 2005).

Excitotoxicity

Excitotoxicity was first described by Olney in the 1970s (Olney, 1969). It consists in a pathological process where an overactivation of receptors for the excitatory neurotransmitter glutamate leads to neuronal death. In physiological conditions, the presence of glutamate in the synapse is regulated by active ATP-dependent transporters in neurons and glia. The induction of excitotoxic neuronal death depends on Ca^{2+} influx through NMDA receptors (Choi, 1992; Limbrick et al., 2001; Randall and Thayer, 1992). In physiological conditions, Ca activates a number of Ca^{2+}-dependent enzymes that influence a wide variety of cellular components, like cytoskeletal proteins or second messenger synthases. However, overactivation at NMDA receptors triggers an excessive entry of Ca^{2+}, initiating a series of cytoplasmic and nuclear processes that promote neuronal cell death: activation of proteolytic enzymes, activation of Ca2+/calmodulin kinase II (with increased activity of phosphorylated enzymes), activation of

Ca^{2+}-dependent endonucleases, and expression of transcription factors such as c-Fos, c-Jun or c-Myc. Mitochondrial dysfunction resulting from pathologic receptor activation plays a central role in the delayed necrotic death of the neuron (Budd and Nicholls, 1996; Wang and Thayer, 1996). In fact recent findings have shown that the initial Ca^{2+} loading into the cytoplasm and Ca elevation are not neurotoxic per se, as long as the mitochondria are depolarized (Stout et al., 1998). In contrast, the further uptake of intracellular Ca^{2+} into the mitochondria creates a condition that results in an irreversible failure of cytoplasmic Ca^{2+} extrusion (Limbrick et al., 2001); this delayed failure of cytoplasmic Ca^{2+} homeostasis seems to be involved in activation of neuronal death pathways (Casthilo et al., 1999; Nicholls et al., 2007).

Neuroprotective properties of cannabinoids

Brain injury results in neurodegenerative events within the nervous system; in fact, traumatic events like stroke, physical trauma, inflammatory reactions, can trigger neurotoxic cascades, ultimately leading to neuronal death. Excitotoxicity takes center stage in the pathologic sequelae after stroke or traumatic brain injury (DeLorenzo et al., 2006; Lipton, 1999; Siesjö, 1992; Siesjö and Bengtsson, 1989), and has been implicated in the slow progression of neurodegenerative disorders such as multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and Huntington's disease (Kim et al., 2002; LaFerla, 2002; Mattson et al., 2000; Missiaen et al., 2000).

The events that lead to excitotoxic cell death can be initiated at many levels: by ATP depletion secondary to oxygen and glucose deprivation; by mitochondrial disorders (Pang and Geddes, 1997); by exogenous administration of glutamate receptor agonists (Dijkhuizen et al., 1996); by removing the voltage-sensitive Mg^{2+} blockade from the NMDA-receptor (Zeevalk and Nicklas, 1992); by blocking glutamate-uptake (Velasco et al., 1996); by pharmacologically inhibiting Na^{+}/K^{+}-ATPase thereby directly

inducing depolarisation, etc. Regardless of the point of initiation, the neurotoxic events self-amplify, and ultimately lead to cell death. Several mechanisms are considered the rational basis of neuroprotective properties of cannabinoids (Drysdale and Platt, 2003):

a) reduction and regulation of transmitter release ;
b) modulation of calcium homeostasis and excitability;
c) antioxidant properties;
d) modulation of immune response.

Cannabinoids showed neuroprotection against brain injury resulting from injected toxins, hypoxia and head trauma: endocannabinoids (AEA and 2-AG) synthesis is strongly enhanced after brain injury, and there is evidence that these compounds reduce the secondary damages (reviewed in Mechoulam et al., 2002; van der Stelt et al., 2002). Some plant and synthetic cannabinoids (CBD, HU-211), have also been shown to be neuroprotective with mechanisms different from the cannabinoid receptors binding, possibly through their direct effect on the excitatory glutamate system and/or as antioxidants (Mechoulam et al., 2002). Researchers have found protective effects from endogenous cannabinoids in models of multiple sclerosis (Baker et al., 2001; Croxford et al., 2008; Ligresti et al., 2006). Anandamide levels in the brains of rats rise after kainate administration and protect against excitotoxicity (Marsicano et al., 2003), and the cannabinoid system may play a primary role in limiting brain damage (Mechoulam and Lichtman, 2003). The mechanisms by which the cannabinoids reduce damage to the brain are related to enhanced GABAergic tone, reducing glutamate activity, as well as to inhibition of nitric oxide and TNFα production (Molina-Holgado et al., 1997). Neuroprotective effects of the CB_1 agonists WIN55212-2 and CP55940 have also been demonstrated against neuronal death induced by glutamate *in vitro*, via CB_1 receptor (Hampson and Grimaldi, 2001; Shen and Thayer, 1998). Similar results are reported with the cannabinoid agonist WIN55212-2 inhibiting glutamate release in an *in vivo* model of hypoxia-

ischemia in newborn rats. This effect is mediated by both CB_1 and CB_2 receptors (Martinez-Orgado et al., 2003; Fernández-López D et al., 2007). WIN55212-2 is also known to inhibit certain calcium channels, (Hampson and Grimaldi, 2001), and the production of cytokines (Sheng et al., 2005). Neuroprotective effects of THC and CBD, and of CB_1 agonists may also involve their antioxidant properties. (El-Remessy et al., 2003; Hampson et al., 1998; Marsicano et al., 2002).

Some *in vitro* and *in vivo* studies do not support a neuroprotective action of cannabinoids (van der Stelt et al., 2002).

Cannabinoids and epilepsy

Epilepsy is one of the most common diseases of the brain, characterized by the periodic and unpredictable occurrence of epileptic seizures, which are caused by an abnormal discharge of cerebral neurons. Many different types of seizures can be identified on the basis of their clinical phenomena. These clinical characteristics, along with their electroencephalographic (EEG) features, can be used to categorize seizures in partial and generalized ones. Partial focal, local seizures have a localized onset in a portion of one hemisphere, while generalized seizures are those in which evidence for a localized onset is lacking. In the absence of a specific etiological understanding in any of the epilepsies or epileptic syndromes, approaches to drug therapy of epilepsy must necessarily be directed at the control of symptoms, i.e. the suppression of seizures (Löscher, 1997). In fact, all currently available drugs are anticonvulsant - antiseizure rather than antiepileptic. In most patients with epilepsy the prognosis for seizure control is very good. Since a significant proportion of individuals with epilepsy suffer from pharmacoresistant epilepsy, there is a clear need for new drugs or new strategies of therapeutic management (Löscher and Schmidt, 2002), and also new drugs with benefits in terms of side effects and tolerability are needed (Schmidt and Kramer, 1994). Relatively high densities of CB_1 receptors are

localized in areas such as the hippocampus, amygdala, and cerebral cortex, areas known to subserve various kinds of seizures (Adams et al., 1997).

Thorough investigations on the anticonvulsant activity of phytocannabinoids were made during the 1970s, around one decade after CBD and THC had been isolated from Cannabis sativa and characterized, but before the endocannabinoid system had been discovered. CBD (and cannabinol) showed to possess an anticonvulsant activity comparable to that of THC in the maximal electroshock test in mice (Karler et al., 1973; Karler et al., 1974a,b), and tolerance to this activity developed only for THC. Pure THC was compared to clinically used anti-epileptic drugs (diphenylhydantoin, phenobarbital and chlordiazepoxide) demonstrating that it increased the latency of tonic convulsion in the pentylentetrazol-induced seizures in mice, but along with diphenylhydantoin at high dosages enhanced the effect of the chemoconvulsant (Sofia et al., 1976). During the 1980s small clinical trials were made to assess therapeutic effect of CBD in patients suffering from epilepsy. “Beneficial effect” in patients refractory to all known antiepileptic drugs was reported, and CBD was considered acting as an antiepileptic drug with no sign of toxicity (Carlini and Cuhna, 1981). In the same period, the three-dimentional structures of CBD and phenytoin were compared and both drugs showed to be “in line with the stereochemical requirements suggested for anticonvulsant drug action” (Tamir et al., 1980). Later CBD was suggested to act preferentially to reduce the spread of seizures activity irrespective to their focal origin in the CNS (Consroe et al., 1982). Many studies on CBD, THC, and their analogs in animal seizure models have been performed, showing that they are largely inactive in animal models of absence seizures produced by electroshock or chemoshock methods (Consroe, 1998). On the other hand, they are generally effective against partial seizures produced by topical application of convulsant metals, limbic seizures produced by kindling, and generalized maximal seizures induced in genetically epileptic animals or produced in animals by electroshock or

GABA-inhibiting drugs (Consroe and Snider, 1986). Anticonvulsant mechanisms of phytocannabinoids appear to involve a reduction of the spread of the generalized maximal seizures and, in limbic seizure models, an increase in the threshold for seizures. The anticonvulsant effects of CBD seem not to be stereoselective, and devoids of toxic effects, whereas THC seems to show stereoselectivity, and in some model shows proconvulsant or convulsant activity (Mechoulam et al., 1992).

This pro-convulsive activity might be explained by the fact that CB_1 receptors expressed on inhibitory GABAergic neurons are activated by THC, leading to a decreased release of GABA, and to the increase in seizure susceptibility. On the other hand, CB_1 receptors expressed on excitatory glutamatergic neurons mediate the anti-convulsive activity of endocannabinoids (Lutz, 2004); moreover, systemic activation of CB_1 receptors by exogenous cannabinoids is anti- or pro-convulsive, depending on the seizure model used (Mechoulam and Lichtman, 2003). A promising strategy to alleviate seizure frequency might be the enhancement of endocannabinoid levels by inhibiting the uptake and the degradation of these endogenous compounds (Lutz, 2004).

AIM OF THE STUDY

In vivo

During the XIX century, western medicine adopted the use of Cannabis sativa for its antiemetic, analgesic and anticonvulsant properties. The psychoactive effects of its preparations have been largely ascribed to the presence of Δ-9-tetrahydrocannabinol (THC), but it is also well known that other cannabinoids exhibit a range of pharmacological activities. One of the more widely investigated cannabinoids is cannabidiol (CBD), which greatly contributes to the attenuation of the side effects of THC, when co-administered to patients. The aim of the *in vivo* study was to assess the potential anticonvulsant activity of three different extracts of hemp aerial parts, harvested by genetically selected genotypes (one rich in THC, one rich in CBD, and one devoid of cannabinoids).

In vitro

Excitotoxicity, caused by the excess activation of glutamate receptors and subsequent accumulation of intracellular Ca^{2+}, initiates a cascade of events that ultimately leads to neuronal death and thus takes centre stage in many CNS disorders such as stroke, traumatic brain injury and neurodegenerative diseases. Intracellularly, mitochondria provide ATP as the source of cellular energy and maintain Ca^{2+} levels. Hence, alterations of the mitochondrial physiology cause irreversible neuronal injury due to the inability to sustain homeostasis. The aim of the *in vitro* study was to investigate the possible protection offered by the non-psychoactive cannabinoid cannabidiol (CBD) and the non-competitive NMDA receptor antagonist memantine in models of excitotoxicity and mitochondrial dysfunction.

IN VIVO METHODS

Plant material and extracts

Cannabis sativa L. aerial parts, harvested by three different genotypes, were kindly supplied by the ISCI (Research Institute for Industrial Crops, C.R.A., Via Amendola, 82, I-45100 Rovigo, Italy). One chemovar is rich in Δ-9-tetrahydrocannabinol (THC rich), one chemovar is rich in cannabidiol (CBD rich), one chemovar devoids of cannabinoids (cannabinoid-free). 100 g of each dried drug was macerated in 2 L of ethanol 95%, at 55 °C, for 12 hours. The ethanolic solution was filtered, concentrated to 100 mL with rotavapor and then filtred with a buchner. The final solution was dried with rotavapor to obtain a resinous extract. The THC- and CBD-rich crude extracts were titrated respectively in Δ-9-THC and CBD by Dr. Grassi G.

Pentylentetrazol (PTZ) induced seizures

To characterize the anticonvulsant activity of the Cannabis extracts, an experimental model for grand-mal seizures was used (Shafaroodi et al., 2004). Male CD1 mice (Harlan, Italy, weighting 25 ± 5 g), were housed under controlled conditions, 12 h light: 12 h dark cycle, 22 °C, 60% humidity. Food and water were supplied ad libitum. Procedures and animal comfort were controlled by the University Veterinary Service. The animals were divided in groups ($n \geq 8$) and treated i.p. with the drugs or extracts under investigation. After 60 minutes they were all injected with the chemoconvulsant PTZ (85 mg/kg i.p.). Each animal was placed in an individual cage (this procedure is necessary to avoid aggregation effects on the animals behaviour) (Löscher et al., 1991) and observed for 30 minutes (cut off time). Behavioural responses to PTZ injection (see description in Table 1) were carefully recorded to evaluate the incidence and the latency of generalized convulsions and lethality.

Table 1. Behavioural responses to PTZ injection.

No change
Abnormal behaviour (tremors, scratching, chewing)
Single myoclonic jerks
Atipical convulsions (clonic convulsions involving head or forelimb)
Generalized tonic-clonic convulsions < 5"
Generalized tonic-clonic convulsions ≥ 5"
Death

Extracts and drugs administration

Three dosages of the different Cannabis extracts were investigated in this model: THC rich extract, 10, 25, 50 mg/kg (the dose refers to the concentration of THC in the extract); CBD rich extract, 100, 200, 300 mg/kg (the dose refers to the concentration of CBD in the extract); cannabinoid-free extract, 300, 400 mg/kg (the dose refers to the amount of extract suspended in vehicle). They were all suspended in the same vehicle: 10% propylen glycol, 1% tween 80, in saline. Chlordiazepoxide (Sigma) 10 mg/kg was used as positive control, and dissolved in saline.

Data analysis

Data are presented as latency time to onset of generalized tonic-clonic convulsions and latency to lethal effect. A latency of 1800 seconds was counted for animals which did not show generalized convulsions. Statistical analysis was performed using Graphpad Prism (Version 4.01; GraphPad Software, San Diego, CA, USA). Significance of differences between drug or extract treated groups and controls was determined using Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, due to the non-parametric distribution of the data. Differences were considered significant for $P<0.05$. Incidence of death subsequent to PTZ i.p. injection was expressed as number

of animals surviving to the treatment, and percentage of survived. The incidence was compared among groups using Fisher's exact test. $P < 0.05$ was considered the significance level between groups.

IN VITRO METHODS

Cell culture

Hippocampal cultures were prepared from 1 to 3 day old Sprague Dawley rat pups after cervical dislocation, in accordance with Home Office and institute regulations, as described previously (e.g. Drysdale et al., 2006). The brain was quickly removed, hippocampi were isolated, cut out, and placed in ice-cold filtered HEPES buffered solution (HBS composition in mM: NaCl 130; KCl 5.4; $CaCl_2$ 1.8; $MgCl_2$ 1; HEPES 10; glucose 25). Cold tissue was placed in 1 mg/ml type X and XIV protease solution, and chopped into fine pieces. After 40 minutes, the tissue was rapidly washed in HBS, triturated several times, and centrifuged twice. Before the last centrifugation, HBS was replaced with 90% Minimum Essential Medium (MEM; Gibco, Paisley, UK) with 10% foetal bovine serum (FBS; Helena Biosciences, Sunderland, UK) and 2 mM L-glutamine. Cells were then plated, and kept in a humidified incubator at 37ºC and in 5% CO_2. The cultures were allowed to mature for 48 h prior to replacement of MEM with Neurobasal Medium (Gibco, Paisley, UK), supplemented with 2% B27, 2mM L-glutamine, and 25mM L-glutamate. All compounds used here, apart from media and serum, were from Sigma, Poole, UK. This tissue culture procedure produces mixed cultures that contain glia (~60%, of these: oligodendrocytes <10%, microglia <10% and astrocytes 80–90%); and neurones (~40%). Culture dishes were used for experimentation at days 4–10 in vitro (DIV).

NMDA induced excitotoxicity and Ca imaging protocol

For Ca imaging experiments, hippocampal cultures were washed with HBS at room temperature and loaded with the cell-permeable fluorescent calcium indicator Fura-2-AM (10μm, Molecular Probes, OR, USA) for 1 h in the dark. Cultures were perfused with low Mg^{2+} high Ca^{2+} HBS (composition in

mM: NaCl, 130; KCl, 5.4; $CaCl_2$, 5.0; $MgCl_2$, 0.1; HEPES, 10; glucose, 25; compounds from Sigma), maintaining a flow rate of 1–2 mL ⁄ min, with a gravity perfusion system. Ratiometric imaging was performed with imaging systems fitted onto an Olympus microscope. The system used the Improvision software package Openlab (version 4.03) using a DG-4 illumination system (Sutter Instruments Company, CA, USA) and Hamamatsu Orca-ER CCD camera. The ratio of the two wavelengths emitted (340 nm and 380 nm) is directly proportional to intracellular calcium levels in the cells. Thus, following background fluorescence subtraction, this value was plotted for all neurons and glia after identification of regions of interest (ROI), with frames captured every 5 s. A minimum of four experiments were conducted for each group of treatment, each on cells from a different culture. A control dish was conducted at each session of experiment to make sure that the daily response of the culture to the NMDA insult was consistent with the previous experiments.

Excitotoxicity was induced by application of 1 mM NMDA (in the presence of 100 μM glycine, co-agonist of the NMDA receptor) for 5 minutes in low Mg^{2+} high Ca^{2+} HBS solution. The parameters determined were the response to NMDA, the recovery, and the secondary Ca dysregulation (see data analysis section for details). Measurement ended after a washout of 40 minutes after NMDA application.

3-NP intoxication protocol and cell death determination with PI/Calcein kit

Between 4 and 10 DIV, cultures were selected and subdivided into treatment group categories, maintaining uniform culture quality, composition and cell density. Cultures were treated for 72 hrs with 1 mM of the mitochondrial toxin 3-NP (3-nitropropionic acid) in NB medium in the presence or absence of CBD or memantine (see below). All treatment solutions were prepared and allowed to temperature- and gas-equilibrate in the aforementioned incubated environment for a period of at least 30 min. Incubation solutions were

prepared with final concentrations of either memantine or CBD in NB medium. The choice of concentrations was based on earlier experiments performed with these substances. Solutions were adjusted to physiological pH immediately prior to sterile-filtered application to cultures. Dishes were stained with live/dead cell staining kit (purchased from Sigma) Calcein-AM is converted to green fluorescent calcein in viable cells only. Propidium iodide (PI) intercalates with DNA of dead cells only after passing through disordered areas in cell membrane of dead cells only. Images were visualised using an Axioskop 2 plus microscope (Carl Zeiss, Germany) with a 40x phase contrast water immersion objective, and were captured using an AxioCam HRc camera, controlled by AxioVision software (Version 3.1). In order to identify the dish's cellular composition, a brightfield image was initially captured, followed by image acquisition using Rhodamine (for PI) and FITC (for calcein-AM) filters. Free-hand count of Calcein-AM-stained cells led to the determination of the total number of live cells, and the merged transmission image allowed selection of neurons (three-dimensional and halo in phase contrast) versus glia (flat in appearance). Cells with PI staining were then highlighted to illustrate non-viable cells. Cell viability according to cell type was calculated as percentage survival. Each experiment was repeated at least three times, each on dishes from a different culture. Three dishes were used per culture, and three images were snapped for each dish. This ensured that a suitable number of replicates (n = 9 dishes) were sampled for each treatment for statistical analysis. Means and S.E.M. of percentage survival were calculated for each dish, as the average of the three snaps. This value was then exported to Prism.

Drug application

Calcium imaging. NMDA (Ascent), CBD (a gift from GW Pharmaceuticals / Prof. Pertwee), and memantine (Tocris) used in these studies are pure drugs. The eCBD used in the post treatment protocol, is a CBD-rich Cannabis

extract (GW Pharmaceuticals) containing 64.6% CBD and 2.5% Δ-9-tetrahydrocannabinol (THC) (see Ryan et al., 2006 for further details).

The first set of experiments consisted in a 10 minutes perfusion with CBD 1μM, 100nM, memantine 10μM, or eCBD 1μM straight after the application of NMDA. In one experiment, CBD 1μM was applied for 5 minutes (instead of 10) straight after the application of NMDA.

The second set of experiments, a 5 minutes perfusion of CBD 1μM or memantine 10μM together with NMDA was applied to the cells. Straight after this, CBD 1μM or memantine 10μM were applied for further 5 minutes without NMDA.

In the last set of experiments, CBD 1μM or memantine 10μM were applied to the cells for 10 minutes straight before the application of NMDA without any other drug.

3-NP toxicity. 3-nitropropionic acid (Sigma), CBD 1μM, 10μM, and memantine 10μM used in these studies are pure drugs. 3-NP 1mM dosage was chosen after a toxicity pilot study on three different dosages (data not shown).

Data analysis

Ca imaging. Data were exported to Excel. Ratiometric values obtained from Openlab were plotted against time, and the time course of the fluorescence for each ROI was analysed. Comparisons of pre-NMDA baseline fluorescence and fluorescence values at the end of the washout were expressed as ratio units (340/380nm). The response to NMDA, CBD, or memantine was determined as percentage change from pre-drug baseline fluorescence (% ΔF/F), the values of the response being taken at the maximum rise of the fluorescence within the 5 minutes of the NMDA application. Recovery of basal levels of $[Ca^{2+}]$ of each ROI was calculated as a ratio between the maximum rise in fluorescence registered within NMDA application minus the

pre-NMDA baseline fluorescence, and the maximum rise in fluorescence registered within NMDA application minus the baseline at the end of the measurement - exact formula used was [(NMDA response - baseline pre-NMDA)/(NMDA response – recovery baseline)]: values between 0.9 and 1.1 were considered full recovery; between 1.1 and 2.0 were considered 50% recovery; values > 2.0 were set as no recovery. These values where then expressed as % of neurons showing the recovery. The working hypothesis assumes that more protective treatment leads to a higher percentage of neurons that show a full or partial recovery. The late amplitude values were calculated as the difference between the fluorescence at the end of the measurement and the pre-NMDA fluorescence. The occurrence of secondary Ca dysregulation was also measured, as it is a sign of excitotoxicity: the neurons showing an increase of $[Ca^{2+}]_I$ after the NMDA perfusion, were counted and the occurrence was expressed as % of neurons showing the dysregulation. The rise in $[Ca^{2+}]_I$ within 15′ from NMDA application was considered as early secondary Ca dysregulation, whereas late secondary Ca dysregulation was the increase in $[Ca^{2+}]_I$ later than 15 minutes from NMDA application.

Statistical analysis was performed using Graphpad Prism (Version 4.01; GraphPad Software, San Diego, CA, USA). Normality tests on raw data and on % ΔF/F responses, confirmed absence of normal distribution of data. Therefore, a Kruskal–Wallis test with Dunn's post hoc test was used for group comparisons. For paired comparison Mann-Whitney U test was used. Significance was set at $P < 0.05$.

3-NP toxicity. Statistics were calculated using GraphPad Prism. Mean survival rates (in %) and S.E.M.s were calculated for each group and cell type. Survival rates were calculated for each control group and one-way analysis of variance (ANOVA) was performed for between-group comparisons, followed by post hoc analysis (Dunnett's multiple comparison). $P < 0.05$ values were considered significant.

IN VIVO RESULTS

PTZ induced seizures study

Though Cannabis sativa has been long investigated for its anticonvulsant properties, it is not fully understood whether its possible anticonvulsant activity is due only to the presence of Δ-9-THC, or it can also be attributed to the presence of CBD, or other cannabinoids and non-cannabinoid compounds. In order to investigate this hypothesis an experimental model for grand-mal seizures was used.

When the three different dosages of THC-rich extract were administered to mice (n = 8) 1h before PTZ injection, all animals showed behavioural effects due to THC presence: motor depression and catalepsy, as well as hypothermia. When PTZ 85 mg/kg was injected, 100% of the animals showed generalized convulsions. As it is shown in Fig. 1, THC-rich extract at the dose of 10 mg/kg and 50 mg/kg significantly ($P < 0.01$ and $P < 0.05$ respectively) prolonged the latency for the onset of first generalized tonic-clonic convulsions 8 times. THC-rich at 25 mg/kg significantly ($P < 0.001$) prolonged the latency to first generalized tonic-clonic convulsions more than 10 times; values of latency to onset are summarized in Tab. 2. As it is shown in Fig. 2, THC-rich extract at the dose of 10 mg/kg significantly ($P < 0.05$) increased the survival of the animals after PTZ treatment from 12.5% to 87.5%, and at the dose of 25 mg/kg significantly ($P < 0.01$) increased the survival of the animals after PTZ treatment from 12.5% to 100%.

When the three different dosages of CBD rich extract were administered to mice (n = 8) 1 h before PTZ injection, none of the animals seemed to show signs of Cannabis intoxication, as the extract contained only traces of THC. When PTZ was injected, 100% of the animals showed generalized convulsions. CBD-rich extract 300 mg/kg significantly ($P < 0.01$) prolonged

the latency to first generalized tonic-clonic convulsions (Fig. 1) 8 times. CBD-rich extract at 300 mg/kg significantly ($P < 0.05$) increased the survival of the animals after PTZ treatment, from 12.5% to 75% (Fig. 2).

When the two dosages of cannabinoid-free extract were administered to mice (n = 8) 1h before PTZ injection, none of the animals seemed to show signs of Cannabis intoxication, as this extract contained traces of THC. When PTZ was injected, 100% of the animals showed generalized convulsions, and none of doses were able to protect the animals from the effects of PTZ (Fig. 1 and 2).

When clordiazepoxide 10 mg/kg was administered none of the animals (n =8) showed convulsions or jerks. Latencies of 1800 seconds were counted for this group (Fig. 1, Fig. 2 and Table 2).

Values of latencies to first generalized tonic-clonic convulsion are summarized in Tab. 2.

Table 2. Latencies to first generalized tonic-clonic convulsion following i.p. injection of PTZ 85 mg/kg.

Group of treatment	*Latency to first generalized tonic-clonic convulsion: mean ± S.E.M. (sec)*
Vehicle	113.2 ± 17.07
THC rich 10 mg/kg	953.5 ± 183.6
THC rich 25 mg/kg	1542.0 ± 194.7
THC rich 50 mg/kg	876.6 ± 267.3
CBD rich 100 mg/kg	356.6 ± 89.65
CBD rich 200 mg/kg	507.0 ± 192.8
CBD rich 300 mg/kg	906.4 ± 194.7
Cannabinoid free 300 mg/kg	178.6 ± 105.4
Cannabinoid free 400 mg/kg	259.0 ± 144.4
Chlordiazepoxide	1800.0 ± 0.0

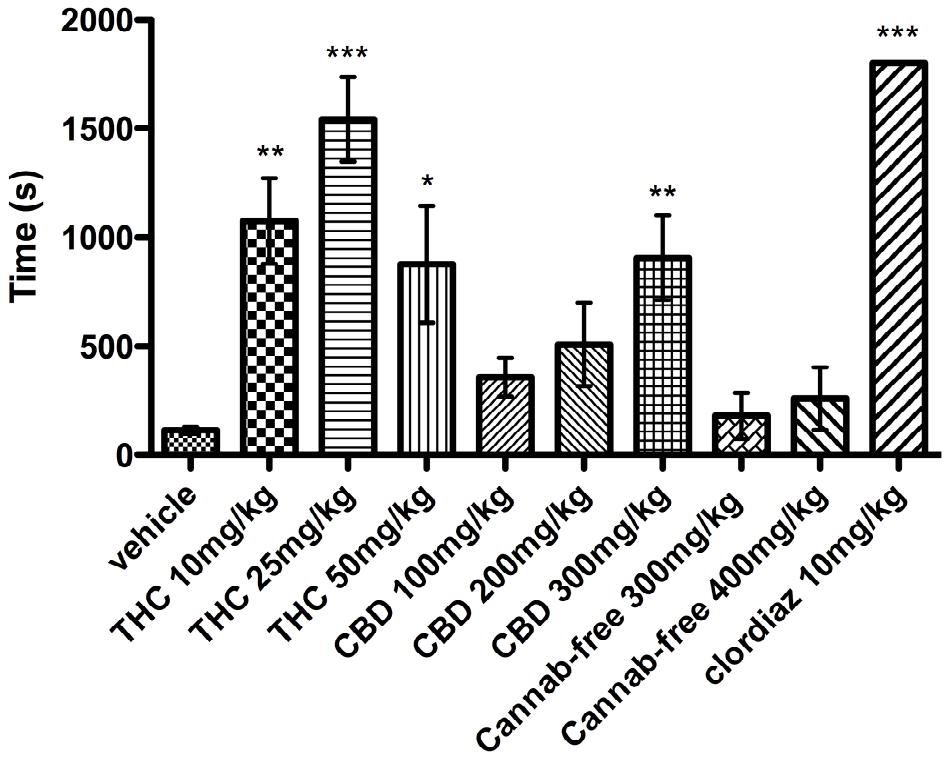


Figure 1. Latency for the onset of first generalized tonic-clonic seizure in animals injected with PTZ 85 mg/kg i.p. and pre-treated with different Cannabis extracts (THC, CBD, Cannab-free) and in control groups. Clordiazepoxide (clordiaz) group represents positive control. Data are presented as mean ± S.E.M. of latency for the onset. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to vehicle group.

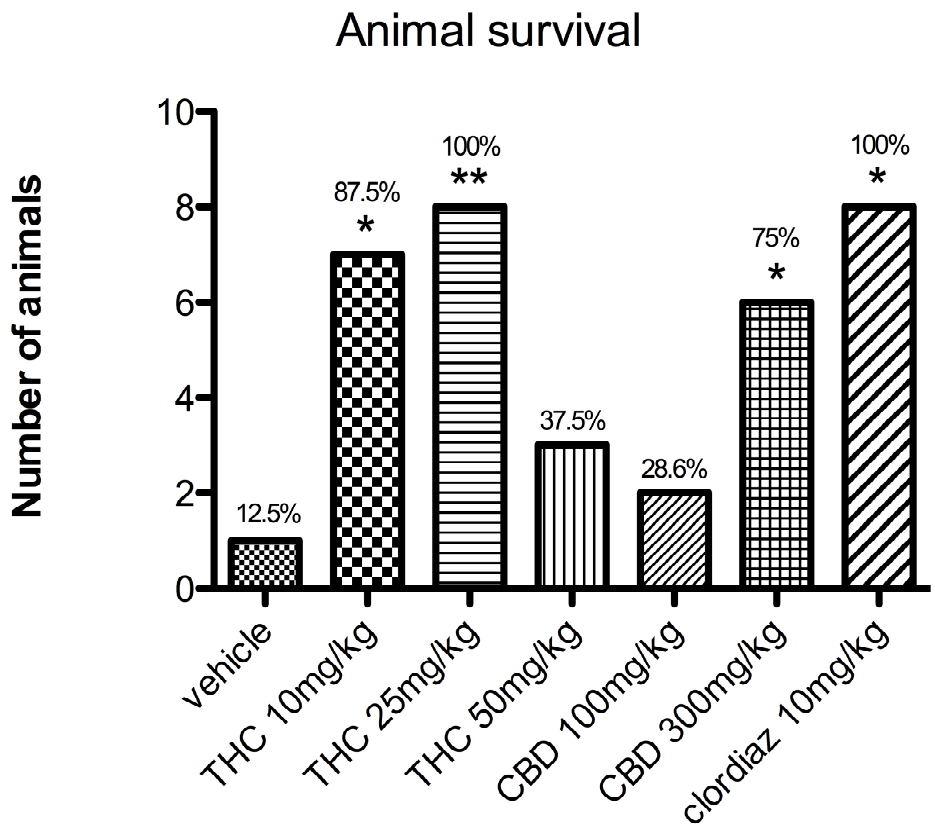


Figure 2. Comparison between lethal effect incidence following i.p. injection of PTZ (85 mg/kg) in animal pre-treated with different Cannabis extracts (THC and CBD) and in control groups, vehicle and clordiazepoxide (clordiaz). The data are presented as number of animals surviving PTZ treatment. (Fisher's exact test *P < 0.05; **P < 0.01). The percentage of mice survived to PTZ treatment for each group, is shown above the columns.

IN VITRO RESULTS

Ca imaging study

The typical response of neurons of a naïve dish to the application of NMDA (1mM) for 5 minutes is shown in Figure 1. The NMDA application provoked an immediate elevation in $[Ca^{2+}]_I$ levels, as represented in the chart by a large increase of the fluorescence. The levels of $[Ca^{2+}]_I$ remained high within the 5 minutes of application, the average response being 339.0 ± 17.2% ΔF/F (n = 124). When NMDA was removed, the fluorescence rapidly dropped to a lower level, and $[Ca^{2+}]_I$ tended to recover to baseline values. During the 40 minutes of washout, some neurons showed a secondary rise in $[Ca^{2+}]_I$. The incidence of this feature was calculated and expressed as secondary Ca dysregulation: the percentage of neurons showing early secondary Ca dysregulation was 13.6 ± 6.2%, whereas 18.9 ± 5.8% of neurons showed late dysregulation (Tab. 1). The percentage of neurons showing full recovery or 50% recovery at the end of the measurement was 1.9 ± 0.9% and 51.1 ± 7.6% respectively; 47.3 ± 7.8% of neurons showed no recovery. At the very end of the measurement only few neurons showed $[Ca^{2+}]_I$ levels similar to pre-NMDA baseline, the late amplitude was 0.58 ± 0.03 ratio units. One neuron (0.8%) showed fluorescence lower than pre-NMDA baseline.

The first set of experiments assessed the ability of CBD and memantine to aid recovery when applied after NMDA. The time course of the fluorescence (Fig. 2), when CBD (1μM) was applied, followed a pattern similar to that of controls: neurons tended to recover to baseline $[Ca^{2+}]_I$ levels within washout time. Some neurons showed a secondary rise in $[Ca^{2+}]_I$ level: in particular, 49.2 ± 16.5% of neurons (n = 97) demonstrated early secondary Ca dysregulation (Fig. 3A) and 12.2 ± 6.5% late Ca dysregulation (Fig. 3B). The percentage of neurons showing full recovery (Fig. 4A) or 50% recovery (Fig. 4B) was 18.7 ± 8.1% and 38.1 ± 9.0% respectively; 43.1 ± 13.2% of neurons

showed no recovery (Fig. 4C). At the very end of the measurement only few neurons showed $[Ca^{2+}]_I$ level similar to pre-NMDA baseline: the late amplitude was significantly ($P < 0.001$) lowered by 24% (from 0.58 ± 0.03 ratio units to 0.44 ± 0.05 ratio units compared to controls; Fig. 5); 4 neurons (4.1%) showed fluorescence lower than pre-NMDA baseline.

When a lower dose of CBD (100nM) was applied (chart not shown) the response of neurons was similar to controls. The 7.7 ± 4.4% of neurons (n = 73) show early secondary Ca dysregulation (Fig. 3A), and 11.3 ± 7.9% of neurons late dysregulation (Fig. 3B). Recoveries to this treatment were characterized by 4.0 ± 3.1% of neurons showing full recovery (Fig 4A), 64.8 ± 10.6% showing 50% recovery (Fig 4B), and 31.2 ± 11.1% no recovery (Fig. 4C). The late amplitude at the end of washout was 0.58 ± 0.05 ratio units (Fig. 5).

When the time of application was changed and CBD (1μM) was applied for 5′ (chart not shown), 3.5 ± 2.2% of neurons showed early secondary Ca dysregulation (Fig 3A), and 30.0 ± 15.5% of neurons showed late Ca dysregulation (Fig 3B). The treatment did not help neuronal recovery: none of neurons (n = 42) showed full recovery (Fig. 4A), the percentage of neurons showing 50% recovery (Fig. 4B) was 60.8 ± 15.7%, and no recovery was 39.2 ± 15.7% (Fig. 4C). At the end of the washout, the late amplitude was 0.59 ± 0.07 ratio units (Fig. 5).

When eCBD 1μM was applied after NMDA for 10′ (chart not shown), it did not alter the parameters analysed. The percentage of neurons (n = 51) showing early dysregulation (Fig. 3A) was 23.6 ± 6.4%, and 5.4 ± 5.4% showed late dysregulation (Fig. 3B). Full recovery was shown by 5.7 ± 3.5% of neurons (Fig. 4A), and 50% recovery by 68.4 ± 12.7% (Fig. 4B). A percentage of 26.0 ± 13.8% showed no recovery (Fig. 4C). $[Ca^{2+}]_I$ at the end of the washout was characterized by a late amplitude of 0.62 ± 0.10 ratio units (Fig. 5).

The response of neurons to the application of memantine 10μM after NMDA, is shown in Figure 6. The time course of fluorescence did not seem to differ much from that of controls, and neurons tended to recover to baseline $[Ca^{2+}]_I$ levels within washout period with a pattern similar to controls. During the 40 minutes of washout, some neurons showed a secondary rise in the $[Ca^{2+}]_I$ level. The percentage of neurons (n = 46) showing early secondary Ca dysregulation (Fig. 3A) was 17.6 ± 8.1%, whereas 13.8 ± 4.0% of neurons showed late dysregulation (Fig. 3B). The percentage of neurons showing full recovery (Fig. 4A) or 50% recovery (Fig. 4B) was 1.9 ± 1.3% and 56.0 ± 13.0% respectively; 40.9 ± 14.2% of neurons showed no recovery (Fig. 4C). At the very end of the measurement only few neurons showed $[Ca^{2+}]_I$ similar to the pre-NMDA baseline: the late amplitude was 0.44 ± 0.04 ratio units (Fig. 5).

The second set of experiments assessed the ability of CBD and memantine to alter NMDA response and aid recovery when applied contemporary to NMDA.

The response of neurons to the application of CBD (1μM) is shown in Figure 7. The time course of fluorescence showed that the rise in $[Ca^{2+}]_I$ due to NMDA application did not seem to be altered by the presence of CBD (394 ± 24% ΔF/F; Fig. 8). As it is shown in Fig. 9 A and B, the percentage of neurons (n = 86) showing early secondary Ca dysregulation was 15.7 ± 9.5%, whereas 7.9 ± 3.9% of neurons showed late dysregulation. The percentage of neurons showing full recovery (Fig. 10A) or 50% recovery (Fig. 10B) was 2.3 ± 1.5% and 58.8 ± 6.5% respectively; 38.9 ± 6.1% of neurons showed no recovery (Fig. 10C). At the very end of the measurement only few neurons showed $[Ca^{2+}]_I$ level similar to pre-NMDA baseline: the late amplitude was 0.91 ± 0.11 ratio units (Fig. 11); 2 neurons (2.3%) showed a fluorescence lower than pre-NMDA baseline.

In comparison, application of memantine (10μM) along with NMDA, had an effect on the parameters analysed, as this drug is a NMDA receptor

antagonist. As it is shown in the chart in Fig. 12, simultaneous application of memantine dampened the response of neurons to NMDA application. As it is shown in Fig. 8, memantine significantly ($P < 0.05$) lowered the response of neurons ($n = 64$) by 23% (from 339 ± 17% ΔF/F to 262 ± 10% ΔF/F); moreover, memantine effect on NMDA response was significantly ($P < 0.001$) different from that of CBD. Memantine did not alter secondary Ca dysregulation: early dysregulation (Fig. 9A) was 22.8 ± 14.4%, whereas 3.2 ± 1.9% of neurons showed late dysregulation (Fig. 9B). As it is predictable by the effect on NMDA response, memantine significantly ($P < 0.01$) increased the percentage of neurons showing full recovery (from 1.9 ± 0.9% to 29.6 ± 12.2%; Fig. 10A), and significantly ($P < 0.05$) lowered the percentage of neurons that did not recover (from 47.3 ± 7.8% to 11.6% ± 9.6%; Fig. 10C). 50% recovery was not altered by the presence of memantine (58 ± 7.7%; Fig. 10B). The application of memantine significantly ($P < 0.001$) lowered the $[Ca^{2+}]_I$ measured at the end of washout by 79%: the late amplitude dropped from 0.58 ± 0.03 ratio units to 0.12 ± 0.02 ratio units (Fig. 11); memantine effect on late amplitude was significantly ($P < 0.001$) different from that of CBD. One neuron (1.6%) showed fluorescence lower than pre-NMDA baseline.

The last set of experiments assessed the ability of CBD and memantine to alter NMDA response and aid recovery when applied before NMDA insult. The response of neurons to application of CBD (1μM) for 10 minutes, is shown in Figure 13: CBD induced a significant ($P < 0.001$) increase in $[Ca^{2+}]_I$ by 16% (from 0.237 ± 0.002 to 0.2831 ± 0.007; Fig. 14A) in neurons perfused ($n = 64$), with a maximum response of 26.16 ± 2.78% ΔF/F (Fig. 14B). This response to CBD was significantly different ($P < 0.001$; Fig. 14A) from that of memantine, which did not produce any increase in fluorescence when applied to neurons (see below); the intensity of CBD response was also significantly different ($P < 0.001$; Fig. 14B) from that of NMDA, when these drugs were applied to naïve dishes. When NMDA was applied after CBD, the

response of neurons (n = 64) was significantly ($P < 0.001$) lowered by 24% from 658 ± 27 % ΔF/F to 503 ± 23% ΔF/F (Fig. 15). The percentage of neurons showing Ca dysregulation did not seem to be altered by pre-application of CBD: early secondary Ca dysregulation (Fig. 16A) occurred in 1.0 ± 1.0% of neurons, whereas 3.4 ± 2.4% of neurons showed late dysregulation (Fig. 16B). The percentage of neurons showing full recovery (Fig. 17A) or 50% recovery (Fig. 17B) was 8.7 ± 5.6% and 86.3 ± 7.9% respectively; 5.0 ± 2.5% of neurons showed no recovery (Fig. 17C). At the very end of the measurement the late amplitude was 0.28 ± 0.03 ratio units (Fig. 18).

The response of neurons to application of memantine (10μM) for 10 minutes is shown in Figure 19: the drug did not seem to produce any alteration of $[Ca^{2+}]_I$ in neurons (Fig. 14A). On the other hand, when NMDA was applied after memantine, the response of neurons (n = 61) was significantly ($P < 0.001$) lowered by 21% (from 658 ± 27% ΔF/F to 517 ± 25% ΔF/F; Fig. 15). The percentage of neurons showing early secondary Ca dysregulation (Fig. 16A) was 4.4 ± 4.4%, whereas 2.5 ± 2.5% of neurons showed late dysregulation (Fig. 16B). The percentage of neurons showing full recovery is 34.1 ± 14.1%, and percentage of neurons showing 50% recovery was 56.2 ± 11.9%; 9.7 ± 4.2% of neurons show no recovery (Fig. 17). Memantine significantly ($P < 0.001$) lowered the $[Ca^{2+}]_i$ measured at the end of washout by 48%: the late amplitude dropped from 0.29 ± 0.03 ratio units to 0.15 ± 0.05 ratio units (Fig. 18); memantine effect on late amplitude was significantly ($P < 0.001$) different from that of CBD. One neuron (1.6%) showed fluorescence lower than pre-NMDA baseline.

Tables 1, 2, and 3 summarize all the effects of CBD and memantine on the different parameters analyzed.

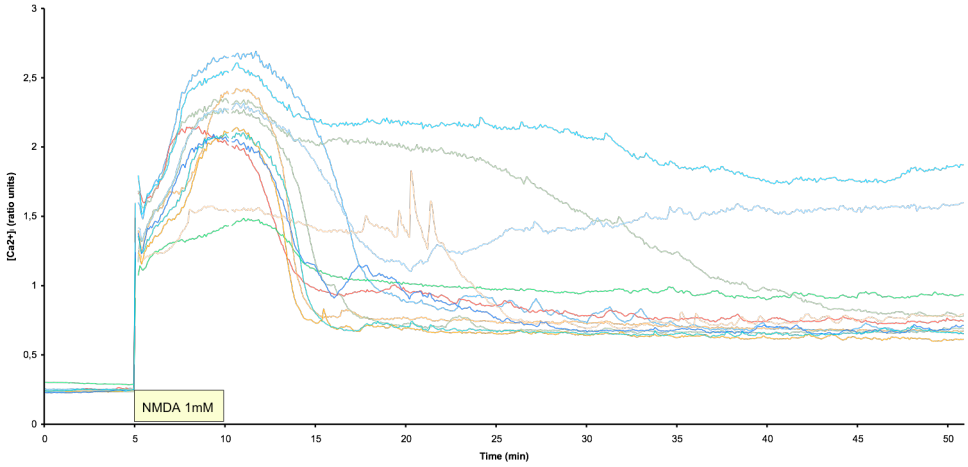


Figure 1. Time course of NMDA induced $[Ca^{2+}]_I$ response in a population of neurons perfused with low Mg^{2+} high Ca^{2+} HBS solution. $[Ca^{2+}]_I$ is expressed as ratio units. Each track represents the time course of one neuron.

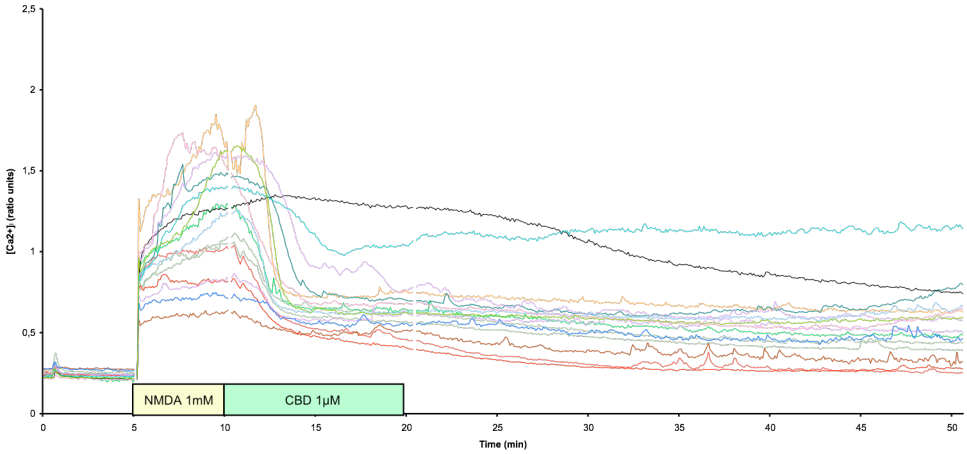


Figure 2. Time course of the $[Ca^{2+}]_I$ response induced by CBD 1μM application after NMDA for 10′, in a population of neurons perfused with low Mg^{2+} high Ca^{2+} HBS solution. $[Ca^{2+}]_I$ is expressed as ratio units. Each track represents the time course of one neuron.

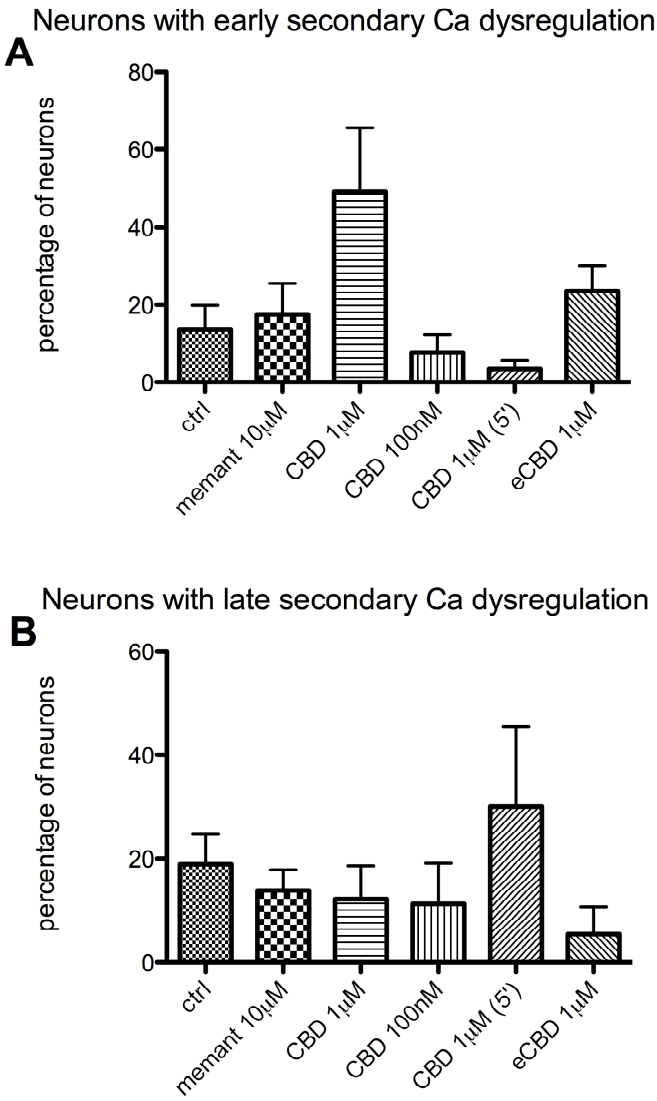


Figure 3. Occurrence of early secondary Ca dysregulation (A) and of late secondary Ca dysregulation (B) in neurons treated with memantine (memant) 10µM, CBD 1µM, 100nM, or eCBD 1µM, applied after NMDA for 10 minutes. In one experiment, CBD 1µM was applied for 5 minutes (instead of 10) as it is indicated by brackets. Data are expressed as average percentage of neurons showing dysregulation.

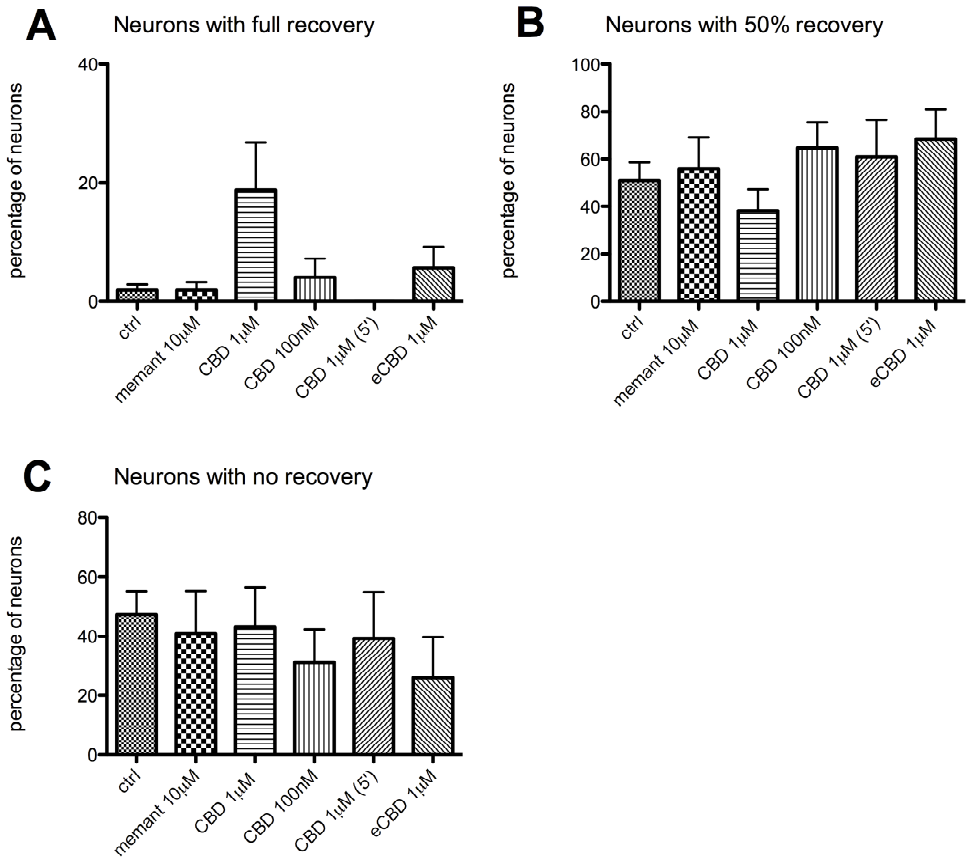


Figure 4. Occurrence of full recovery (A), 50% recovery (B), and no recovery (C) in neurons treated with memantine (memant) 10µM, CBD 1µM, 100nM, or eCBD 1µM, applied after NMDA for 10 minutes. In one experiment, CBD 1µM was applied for 5 minutes (instead of 10) as it is indicated by brackets. Data are expressed as average percentage of neurons showing recovery.

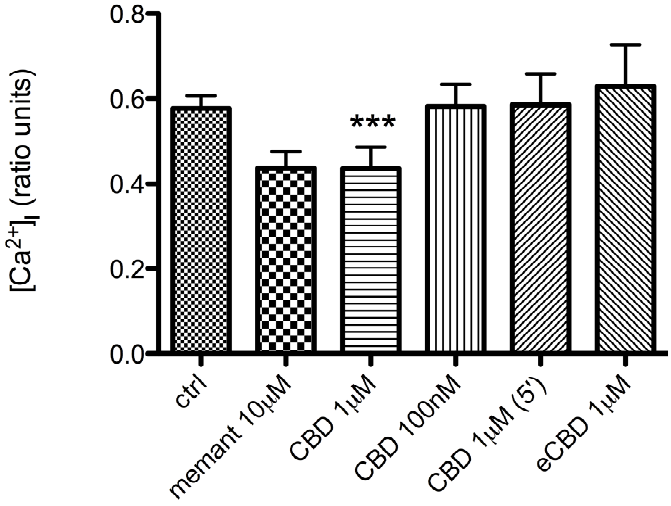


Figure 5. Late amplitude in neurons treated with memantine (memant) 10μM, CBD 1μM, 100nM, or eCBD 1μM, applied for 10 minutes after NMDA. In one experiment, CBD 1μM was applied for 5 minutes (instead of 10) as it is indicated by brackets. Data are expressed as ratio units. *** $P<0.001$ compared with control group using Kruskal–Wallis test with Dunn's post hoc test.

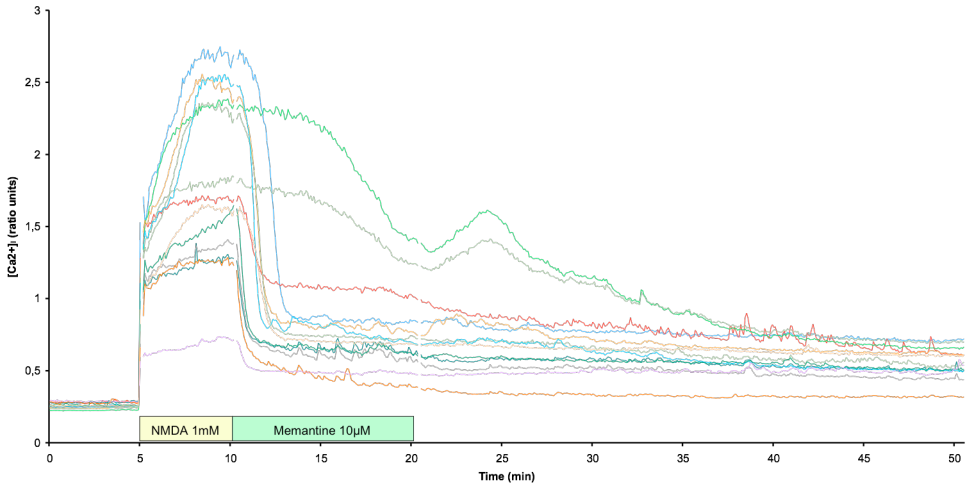


Figure 6. Time course of the $[Ca^{2+}]_I$ response induced by memantine 10µM applied for 10′ after NMDA treatment, in a population of neurons perfused with low Mg^{2+} high Ca^{2+} HBS solution. $[Ca^{2+}]_I$ is expressed as ratio units. Each track represents the time course of one neuron.

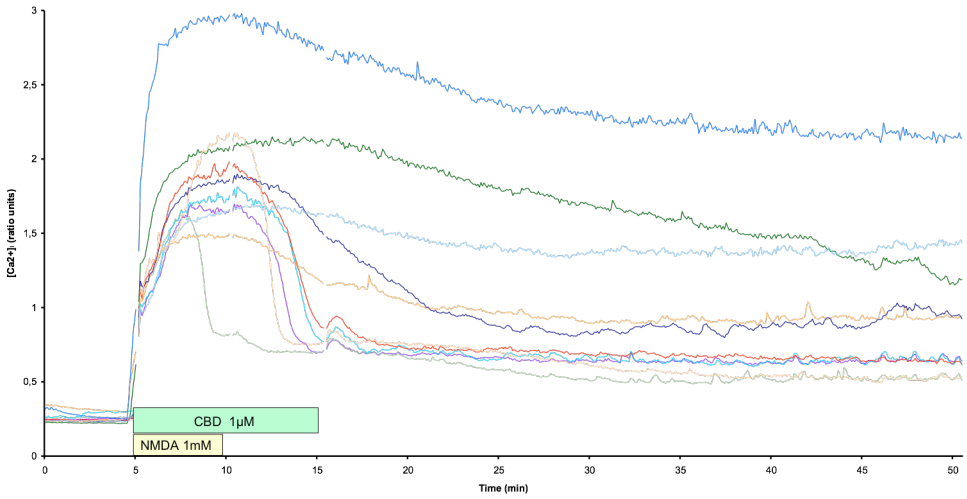


Figure 7. Time course of the $[Ca^{2+}]_I$ response induced by CBD 10µM applied contemporary to NMDA in a population of neurons perfused with low Mg^{2+} high Ca^{2+} HBS solution. $[Ca^{2+}]_I$ is expressed as ratio units. Each track represents the time course of one neuron.

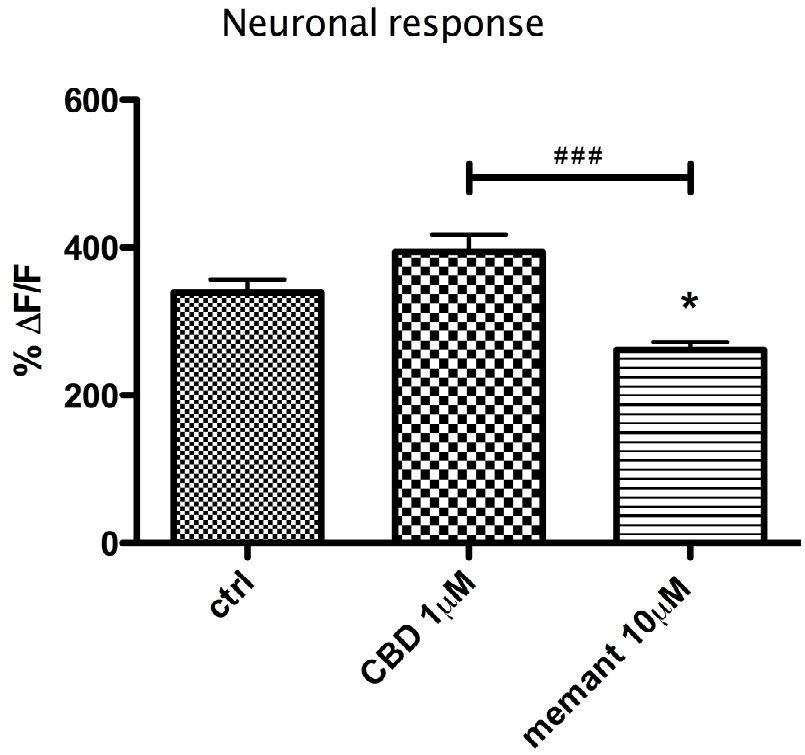


Figure 8. Comparison of NMDA response in control neurons and in neurons perfused with memantine (memant) 10μM, and CBD 1μM contemporary to NMDA application. * $P < 0.05$ compared with control group using Kruskal–Wallis test with Dunn's post hoc test. ### $P < 0.001$ Mann-Whitney paired test.

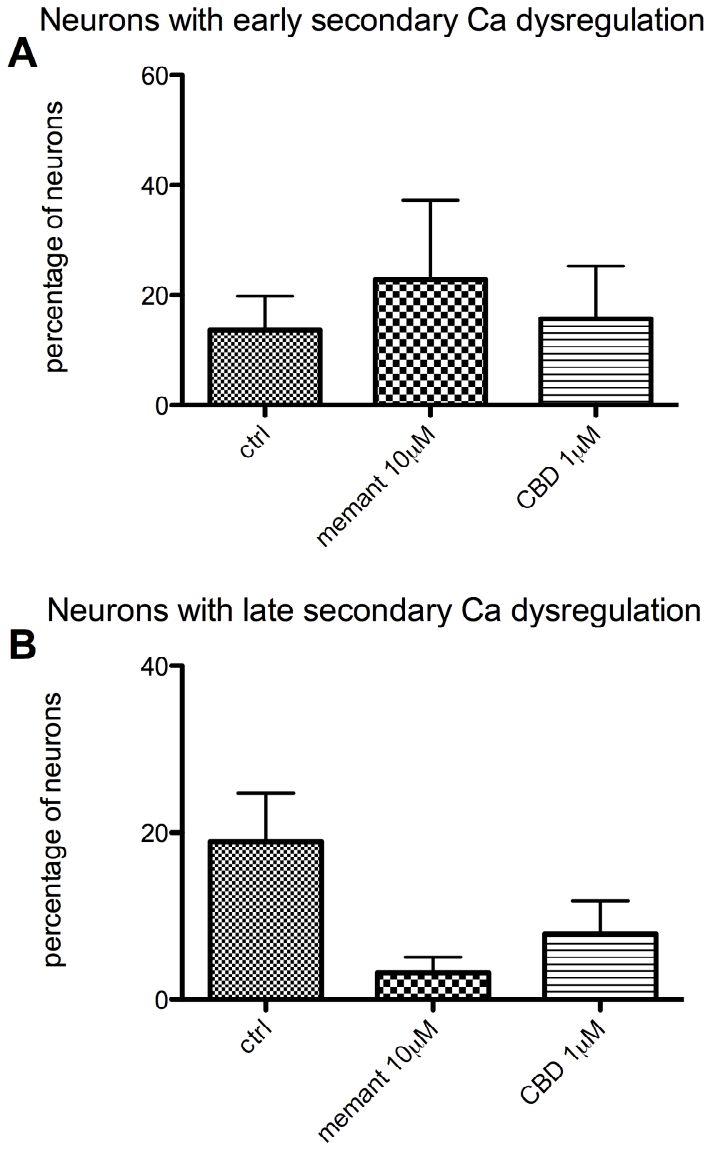


Figure 9. Occurrence of early secondary Ca dysregulation (A) and of late secondary Ca dysregulation (B) in neurons treated with memantine (memant) 10μM, and CBD 1μM, applied contemporary to NMDA. Data are expressed as average percentage of neurons showing dysregulation.

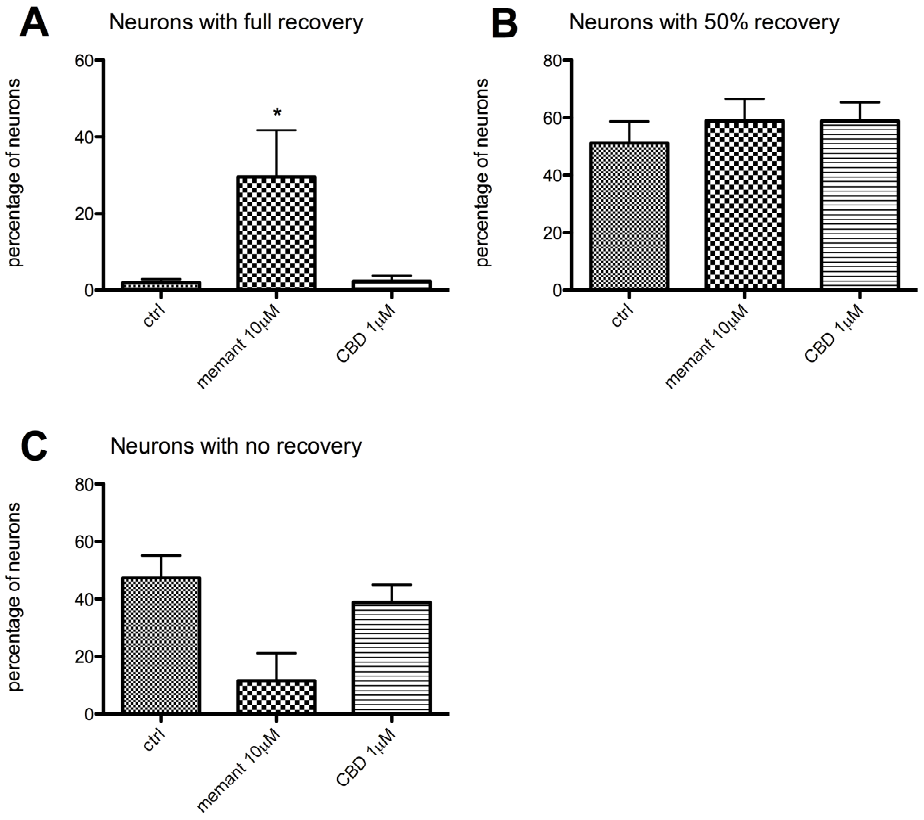


Figure 10. Occurrence of full recovery (A), 50% recovery (B), and no recovery (C) in neurons treated with memantine (memant) 10µM, and CBD 1µM, applied contemporary to NMDA. Data are expressed as average percentage of neurons showing recovery. * $P < 0.05$ compared with control group using Kruskal–Wallis test with Dunn's post hoc test.

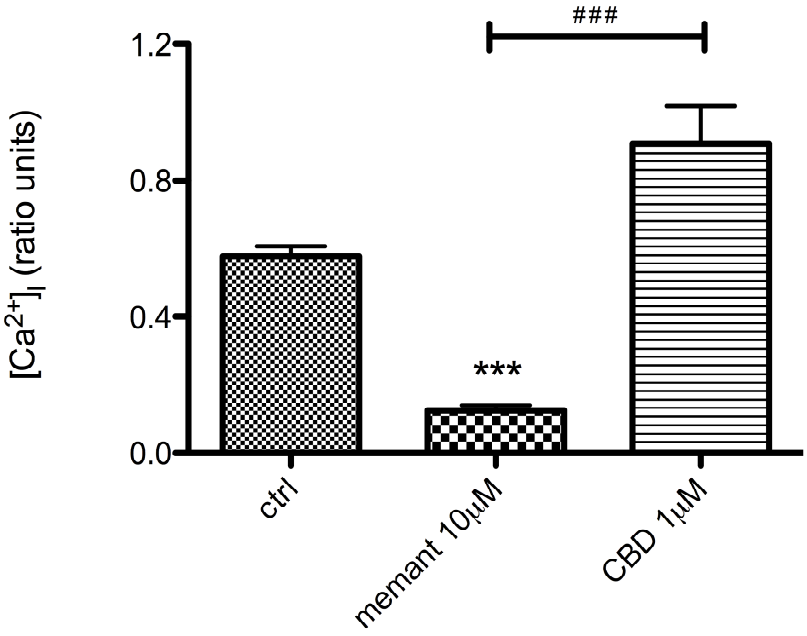


Figure 11. Late amplitude in neurons treated with memantine (memant) 10μM, and CBD 1μM applied contemporary to NMDA. Data are expressed as ratio units. ***P < 0.001 compared with control group using Kruskal–Wallis test with Dunn's post hoc test. ### P < 0.001 Mann-Whitney paired test.

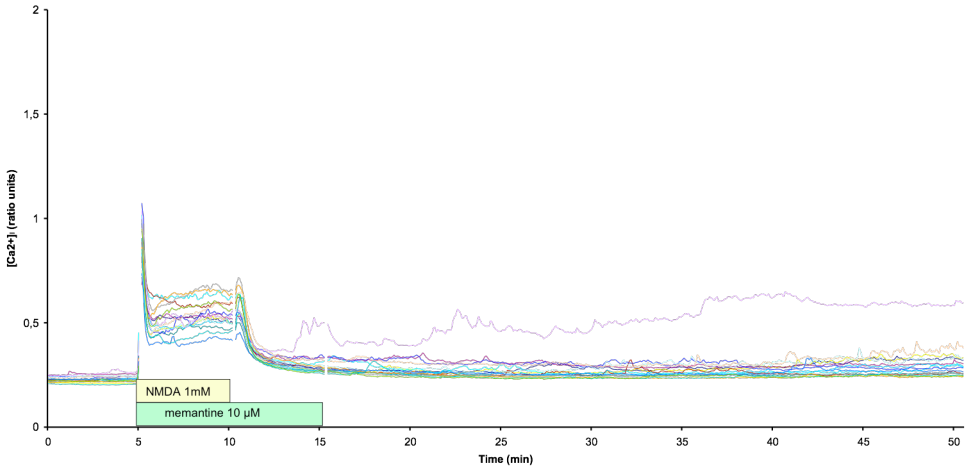


Figure 12. Time course of the $[Ca^{2+}]_I$ response induced by memantine 10µM applied contemporary to NMDA in a population of neurons perfused with low Mg^{2+} high Ca^{2+} HBS solution $[Ca^{2+}]_I$ is expressed as ratio units.

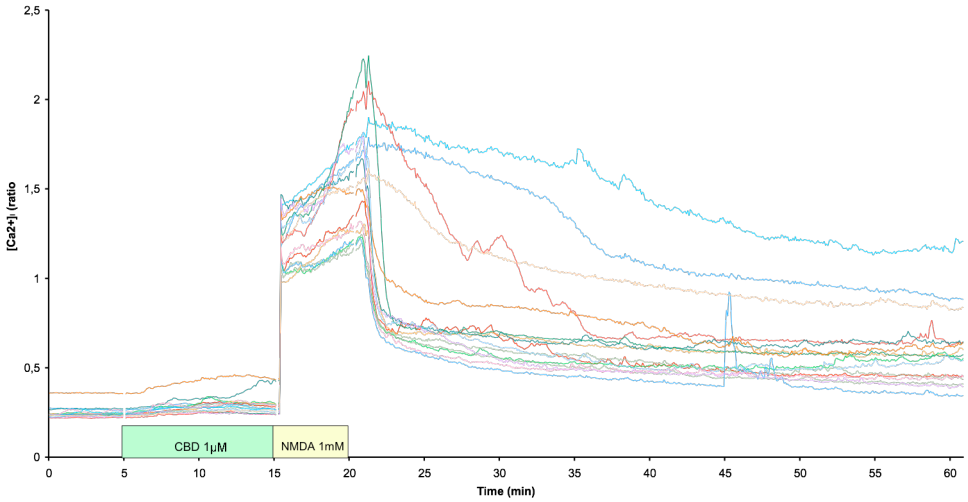


Figure 13. Effect of the $[Ca^{2+}]_I$ response induced by CBD 1µM application before NMDA for 10' in a population of neurons perfused with low Mg^{2+} high Ca^{2+} HBS solution. $[Ca^{2+}]_I$ is expressed as ratio units.

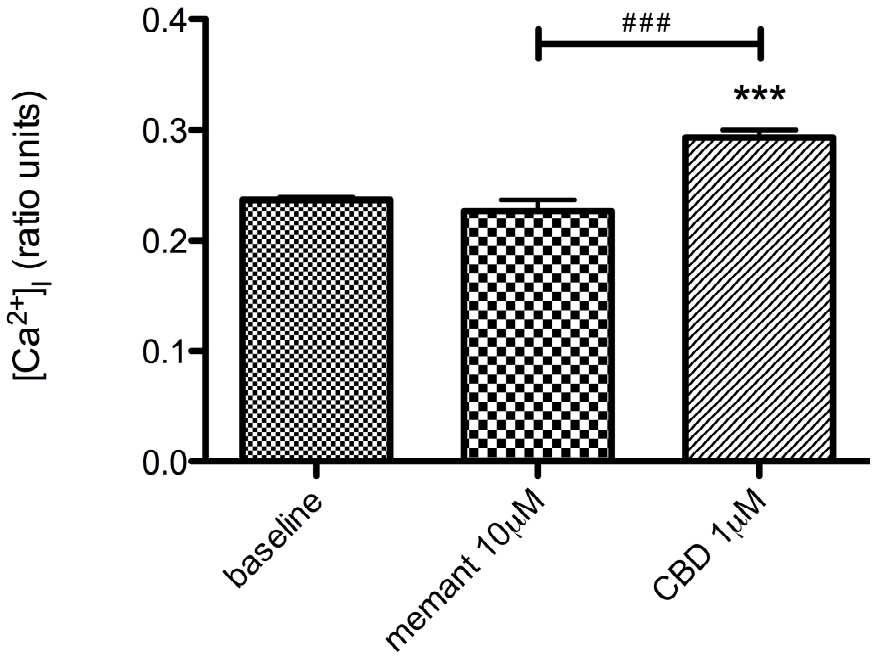


Figure 14A. Comparison of $[Ca^{2+}]_I$ values (expressed as ratio units) in naïve neurons and in neurons perfused with memantine (memant) 10μM, and CBD 1μM. Memantine did not evoke any response in neurons, whereas CBD produced a significant response: *** P<0.001 compared with baseline levels using Kruskal–Wallis test with Dunn's post hoc test. ### P < 0.001 Mann-Whitney paired test.

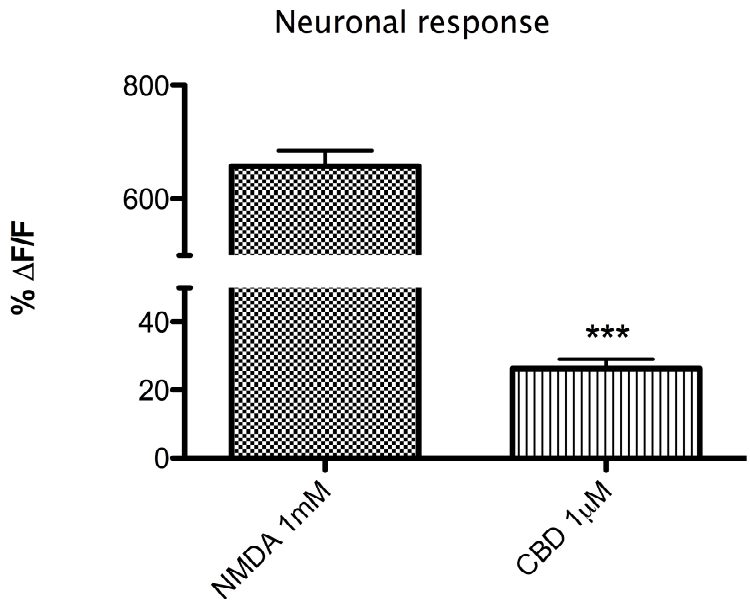


Figure 14B. Comparison between the response produced in neurons by the application of NMDA 1mM and CBD 1μM. *** P<0.001 Mann-Whitney paired test.

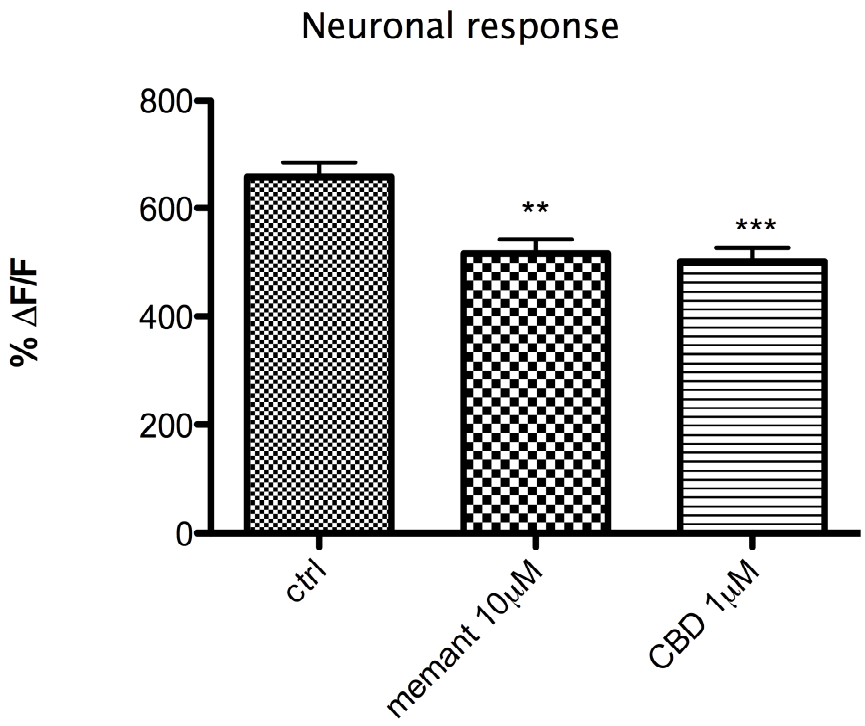


Figure 15. Comparison of NMDA response in controls neurons and in neurons perfused with memantine (memant) 10µM, and CBD 1µM before NMDA application. *** P<0.001, ** P<0.01 compared with control group using Kruskal–Wallis test with Dunn's post hoc test.

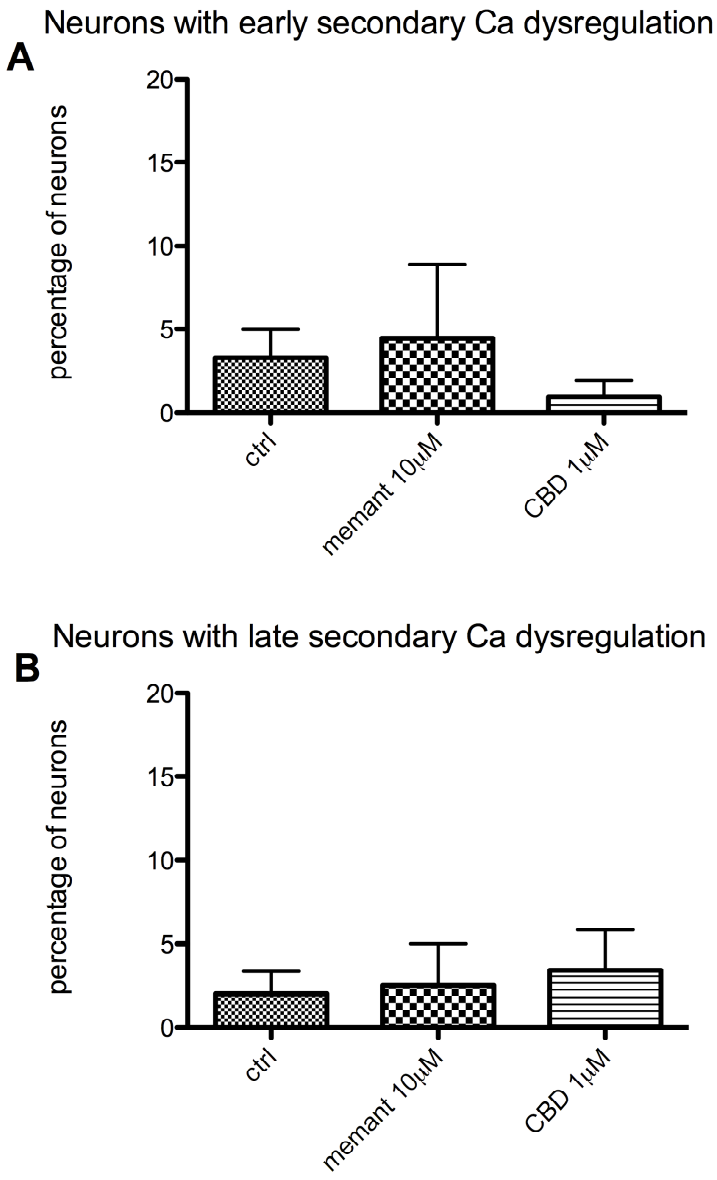


Figure 16. Occurrence of early secondary Ca dysregulation (A) and of late secondary Ca dysregulation (B) in neurons treated with memantine (memant) 10μM, and CBD 1μM, applied before NMDA insult. Data are expressed as average percentage of neurons showing dysregulation.

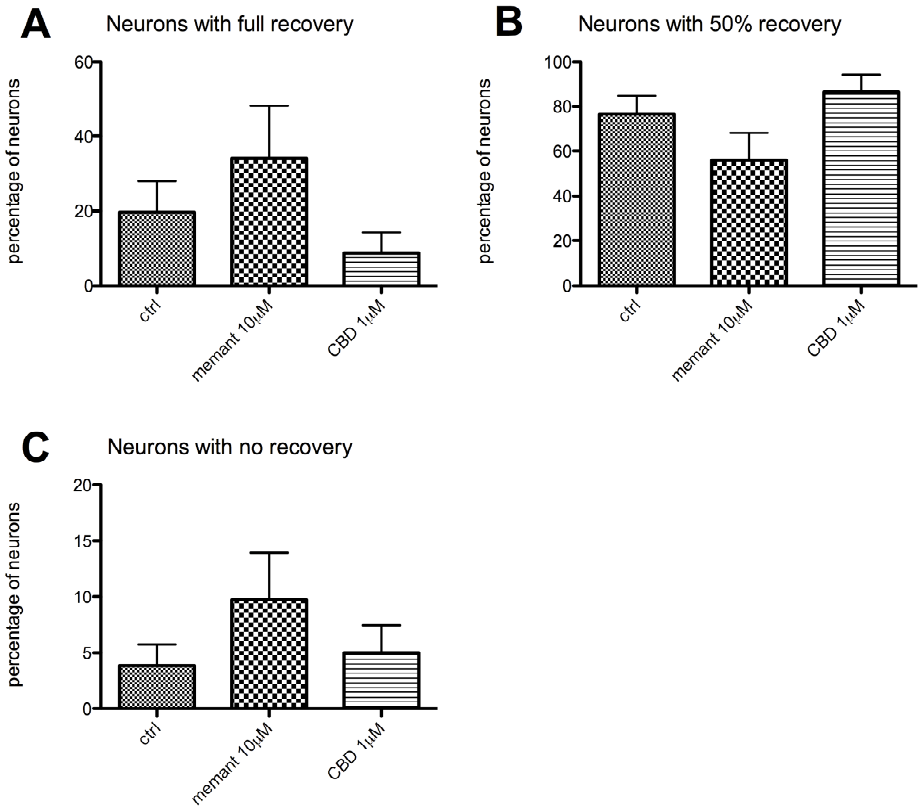


Figure 17. Occurrence of full recovery (A), 50% recovery (B), and no recovery (C) in neurons treated with memantine (memant) 10µM, and CBD 1µM, applied before NMDA for 10 minutes. Data are expressed as average percentage of neurons showing recovery.

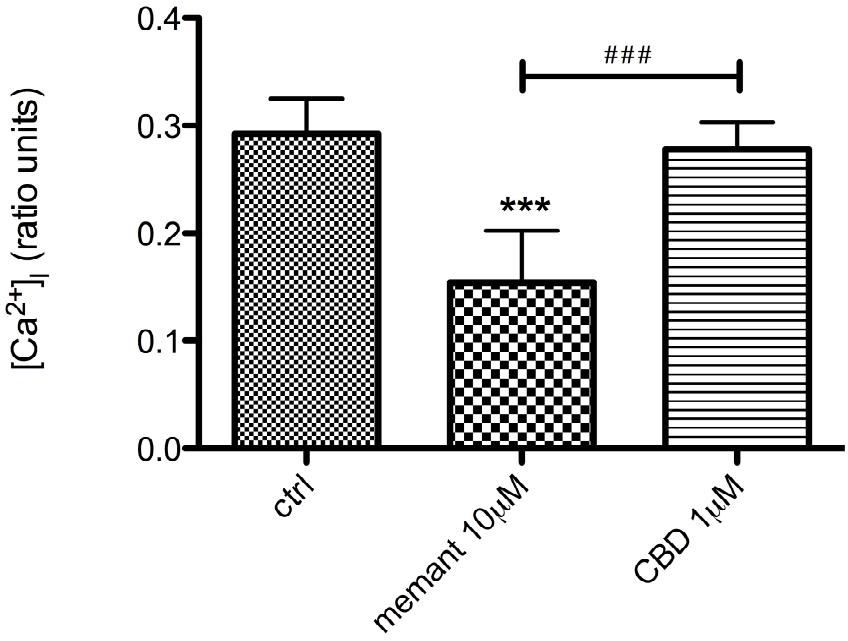


Figure 18. Late amplitude in neurons treated with memantine (memant) 10μM, and CBD 1μM, applied before NMDA for 10 minutes. Data are expressed as ratio units. *** P<0.001 compared with control group using Kruskal–Wallis test with Dunn's post hoc test. ### P < 0.001 Mann-Whitney paired test.

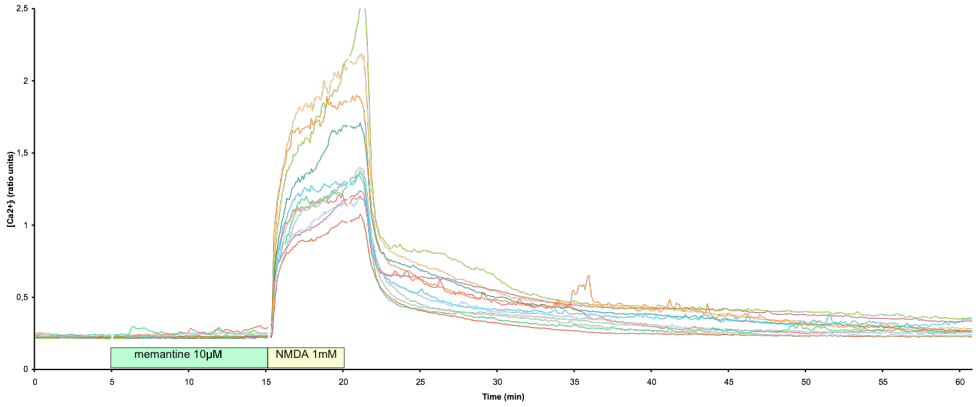


Figure 19. Time course of the $[Ca^{2+}]_I$ response induced by memantine 10µM application before NMDA for 10′ in a population of neurons perfused with low Mg^{2+} high Ca^{2+} HBS solution. $[Ca^{2+}]_I$ is expressed as ratio units.

Table 1. Effects of memantine, CBD, and eCBD on the different parameters analyzed, when they were applied after NMDA.

	control	Memantine 10µM	CBD 1µM	CBD 100nM	CBD 1µM (5')	eCBD 1µM
early dysreg	%	%	%	%	%	%
mean	13.6	17.6	49.2	7.7	3.5	23.6
S.E.M.	6.2	8.1	16.5	4.4	2.2	6.4
late dysreg	%	%	%	%	%	%
mean	18.9	13.8	12.2	11.3	30.0	5.4
S.E.M.	5.8	4.0	6.5	7.9	15.5	5.4
full recovery	%	%	%	%	%	%
mean	1.9	1.9	18.7	4.0	0.0	5.7
S.E.M.	0.9	1.3	8.1	3.1	0.0	3.5
50% recovery	%	%	%	%	%	%
mean	51.1	56.0	38.1	64.8	60.8	68.4
S.E.M.	7.6	13.0	9.0	10.6	15.7	12.7
no recovery	%	%	%	%	%	%
mean	47.3	40.9	43.1	31.2	39.2	26.0
S.E.M.	7.8	14.2	13.2	11.1	15.7	13.8
late amplitude	%	%	%	%	%	%
mean	0.58	0.44	0.44	0.58	0.59	0.62
S.E.M.	0.03	0.04	0.05	0.05	0.07	0.10

Table 2. Effects of memantine, and CBD on the different parameters analyzed, when they were applied simultaneously to NMDA.

	control	memantine 10μM	CBD 1μM
response	% ΔF/F	% ΔF/F	% ΔF/F
mean	339	262	394
S.E.M.	17	10	24
early dysregulation	%	%	%
mean	13.6	22.8	15.7
S.E.M.	6.2	14.4	9.5
late dysregulation	%	%	%
mean	18.9	3.2	7.9
S.E.M.	5.8	1.9	3.9
full recovery	%	%	%
mean	1.9	29.6	2.3
S.E.M.	0.9	12.2	1.5
50% recovery	%	%	%
mean	51.1	58.9	58.8
S.E.M.	7.6	7.7	6.5
no recovery	%	%	%
mean	47.3	11.6	38.9
S.E.M.	7.8	9.6	6.1
late amplitude	ratio units	ratio units	ratio units
mean	0.58	0.12	0.91
S.E.M.	0.03	0.02	0.11

Table 3. Effects of memantine, and CBD on the different parameters analyzed, when they were applied before NMDA.

	control	memantine 10μM	CBD 1μM
response	% ΔF/F	% ΔF/F	% ΔF/F
mean	658	517	503
S.E.M.	27	25	23
early dysreg	%	%	%
mean	3.3	4.4	1.0
S.E.M.	1.7	4.4	1.0
late dysreg	%	%	%
mean	2.0	2.5	3.4
S.E.M.	1.4	2.5	2.4
full recovery	%	%	%
mean	19.7	34.1	8.7
S.E.M.	8.3	14.1	5.6
50% recovery	%	%	%
mean	76.5	56.2	86.3
S.E.M.	8.3	11.9	7.9
no recovery	%	%	%
mean	3.9	9.7	5.0
S.E.M.	1.9	4.2	2.5
late amplitude	ratio units	ratio units	ratio units
mean	0.29	0.15	0.28
S.E.M.	0.03	0.05	0.03

3-NP study

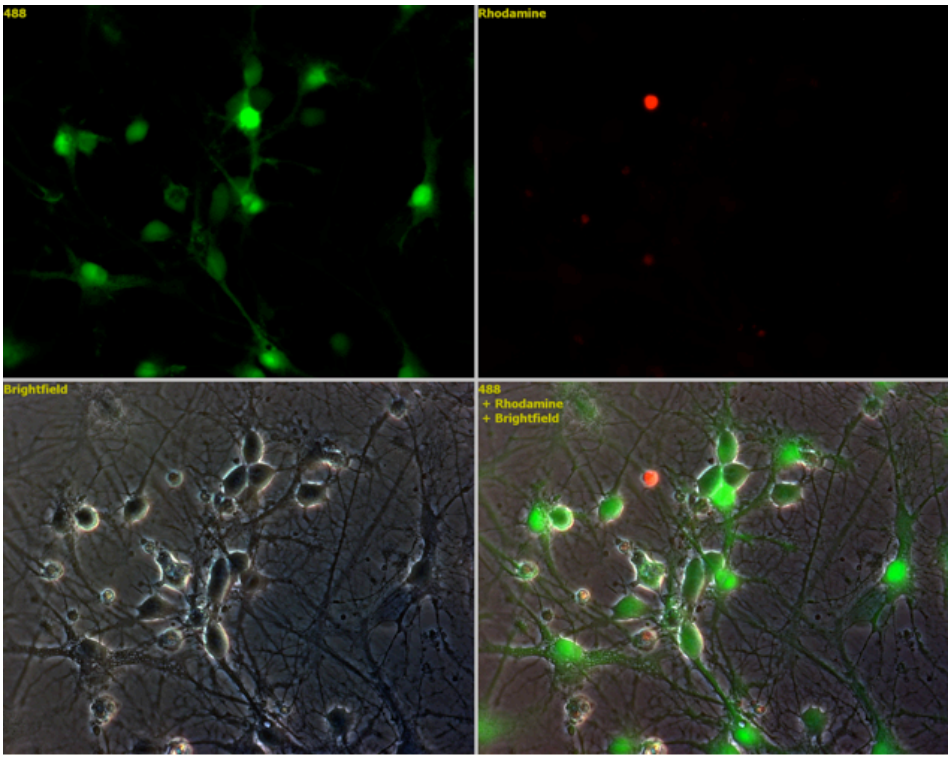
When 3-NP is applied to neurons, mitochondrial metabolism is altered and this leads to impairment of the energetic homeostasis of the cells. 3-NP inhibits succinate dehydrogenase, interferes with the synthesis of ATP, and alters energy dependent processes in cells (Alston et al., 1977; Coles et al., 1979). The transmembrane potential is affected: since there is a decrease of ATP levels, Na^+/K^+ ATPase activity is impaired and this leads to cellular depolarization and activation of NMDA receptors. The resulting increase in Ca influx seems responsible for cell damage (Fink et al., 1996).

In Fig. 20, a naïve dish stained with PI and calcein is presented. As it is shown in A, live cells were stained with calcein green, and in B a red spot was clearly visible, indicating a dead neuron stained with PI. The brightfield image of the area is shown in C: this image helps recognizing neurons from glia during the manual count. In D the merged image gives an example of the morphology of the area snapped. This area showed normal morphology, with round soma, surrounded by a phase-contrast halo, and long and interconnected processes. Neurons were brightly stained (green), somas and processes were well visible within the snapping area, distributed with a good density. Underneath the neuronal layer, glial cells are visible, stained in green, but characterized by a less brilliant intensity of fluorescence, compared to neurons. They are recognizable also because they are not surrounded by halo.

As it is shown in Fig. 21, considerable changes in viability and morphology of neurons were found following treatment with 3NP. The cell body of the neurons appeared less round, more irregular, and processes were shrunken. As it is shown in B and D, many neurons were dead after 72h exposure to the toxin. The area snapped was full of undetectable neurons, and cell debris. The glia underneath did not seem to be altered in morphology.

To determine whether memantine or CBD were able to protect neurons from 3-NP induced damages, the drug was applied to the cultures simultaneously with the toxin. As it is shown in Fig. 22, memantine (10μM) seemed to protect the neurons from the toxic effects of 3-NP. The area snapped showed neurons with normal morphology, with regular soma, surrounded by halo, and long and interconnected processes. As it is shown in Fig. 23 memantine application significantly ($P < 0.05$, $n = 9$ dishes) increased the neuronal survival to 3-NP intoxication by 11% compared to controls (from 64 ± 3% to 75 ± 4%). In agreement with previous studies, glial cells were not affected by the 3-NP application. When CBD (1μM, 10μM) was applied simultaneously with 3-NP (image not shown), it did not show any effect in this experimental protocol ($P > 0.05$; Fig. 23).

A B



C D

Figure 20. PI/calcein stained image captured for analysis; non-treated dish. A. Green transmission, calcein positive staining, live cells. B. Red transmission, PI positive staining, dead cells. C. Brightfield image. D. Merged image of the area.

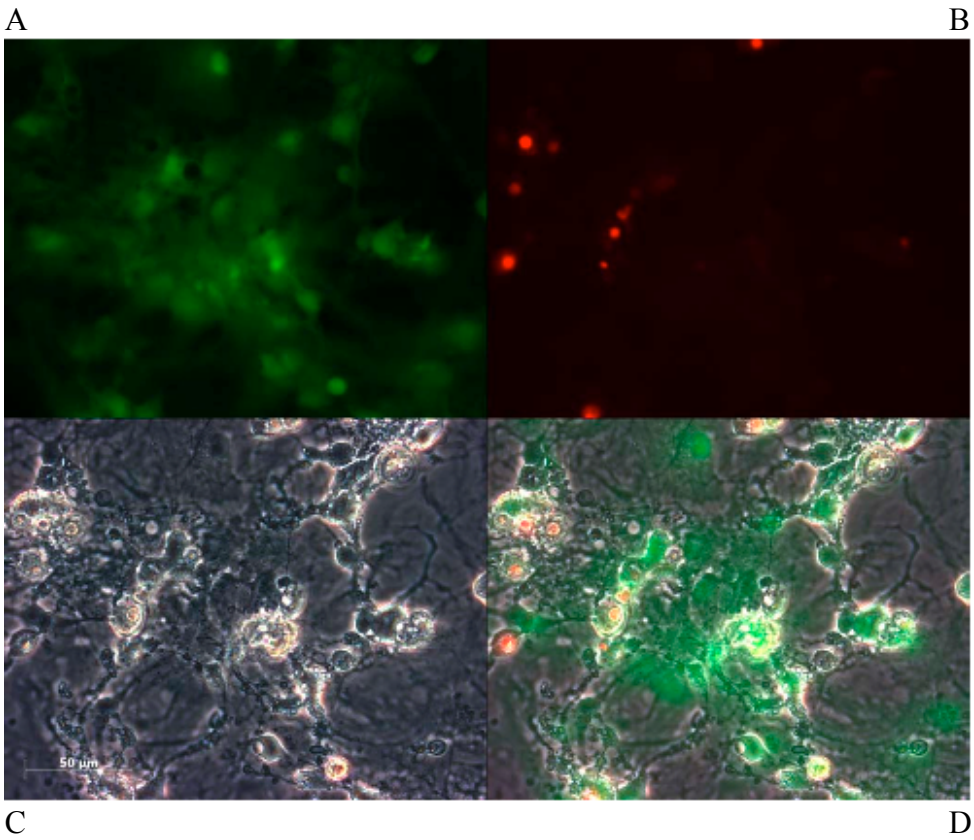


Figure 21. PI/calcein stained image captured for analysis; 3-NP (1mM, 72h) incubated dish. A. Green transmission, calcein positive staining, live cells. B. Red transmission, PI positive staining, dead cells. C. Brightfield image. D. Merged image of the area.

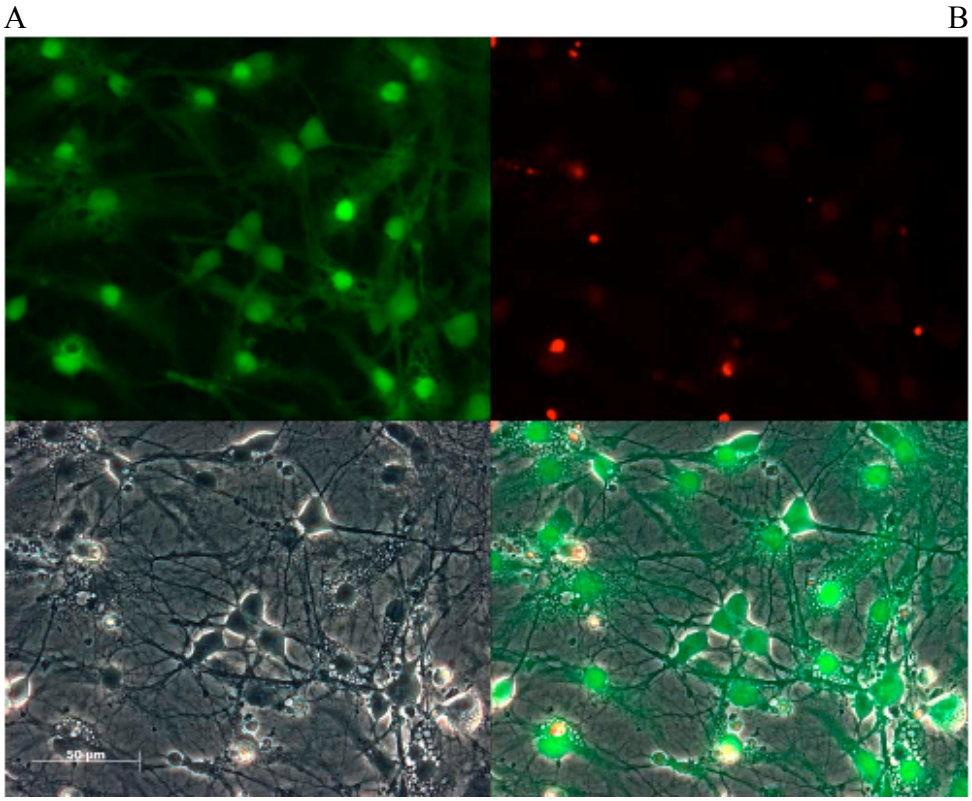


Figure 22. PI/calcein stained image captured for analysis; the dish was treated with memantine 10µM contemporary to 3-NP 1mM, 72h intoxication. A. Green transmission, calcein positive staining, live cells. B. Red transmission, PI positive staining, dead cells. C. Brightfield image. D. Merged image of the area.

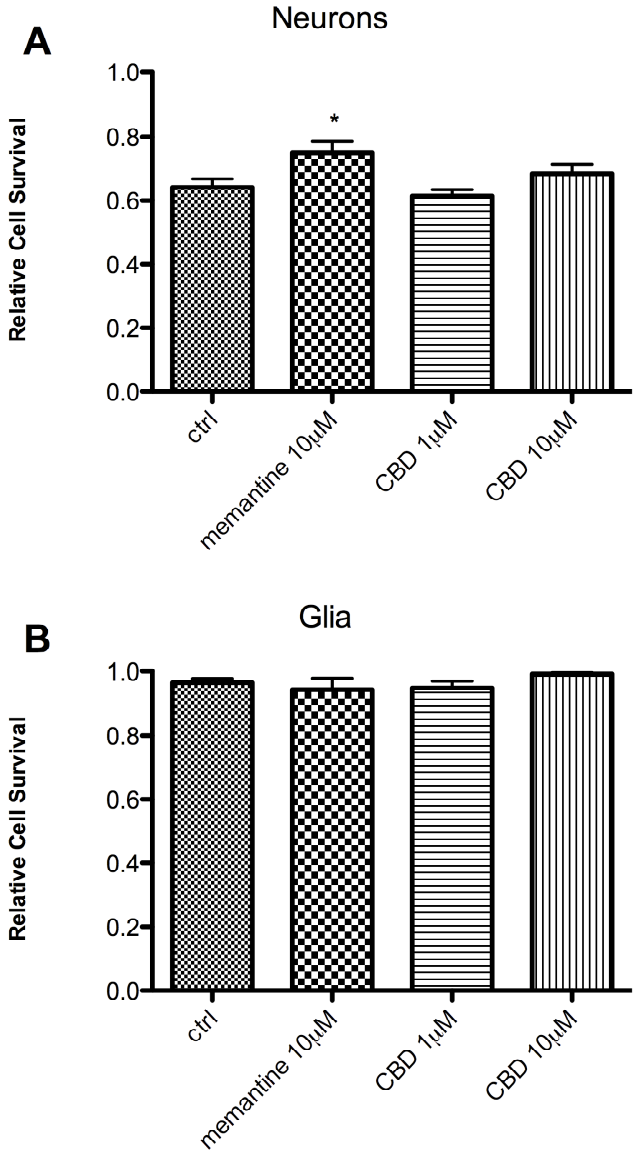


Figure 23. Relative cell survival in neurons and glia intoxicated with 3-NP.
* $P<0.05$ compared with control group using ANOVA followed by post hoc analysis (Dunnett's multiple comparison). Results are mean ± S.E.M. from 9 dishes. Data are expressed as relative cell survival, 100% of survival corresponding to 1.0 relative survival.

DISCUSSION

Discussion of the in vivo study

Cannabis sativa is better known in Western societies for the recreational use of its most popular preparations, marijuana and hashish, whereas its medicinal use has a millennian history (Mechoulam and Ben-Shabat, 1999). The therapeutic potential of Cannabis for the treatment of neurological and psychiatric diseases is currently under investigation. Until a few years ago, the use of THC against emesis and wasting syndrome in patients with terminal diseases seemed the only applications permitted. However, the discovery of THC mechanism of action and of the endocannabinoid system in the 1990s stimulated an increasing number of clinical studies with cannabinoids, as well as on Cannabis extracts. The possible use of cannabinoids in chronic pain, various inflammatory conditions, head injury, glaucoma, epilepsy, and psychiatric disorders is under investigation (Drysdale and Platt, 2003). While the neuroprotective effect of Cannabis extracts is under clinical investigation at the present (Barnes, 2006), and Sativex®, a Cannabis-based medicine containing both THC and CBD, was licensed in Canada as adjunctive treatment for the symptomatic relief of neuropathic pain in patients with multiple sclerosis (Wright, 2007), other therapeutic applications, such as epilepsy treatment, are still under debate. Although Canadian authorities have approved Cannabis-based medicine use in epilepsy patients (Gross et al., 1999), more pre-clinical and clinical evidence are needed to support this indication.

In our experimental protocol we investigated the possible anticonvulsive activity of different Cannabis extracts, in a model of grand-mal seizures. In particular we tested extracts THC- and CBD-rich, and one extract which devoids of cannabinoids. None of them was able to reduce the incidence of seizures in the animals treated with the GABA antagonist PTZ. On the other

hand, both THC- and CBD-rich extracts increased the latency to the onset for generalized tonic-clonic seizures, thus suggesting that they might play a role in the mechanism of generalized seizures triggering (Brevard et al., 2006). The dose-response of THC-rich extract on the latency and on animal survival was bell shaped; the most significant effect was shown by the medium dose. The fact that a higher dose of THC-rich extract was less effective than the medium one may be explained by the fact that there might be compounds in the mixture of the extract acting as antagonists, thus diminishing the action of THC (Pertwee, 2008; Pertwee et al., 2007; Thomas et al., 2005). Another reason is that THC activates CB_1 receptors, but it can also act as an antagonist on those receptors, and this effect may lead to an impairment of the neuroprotective effect mediated by the endocannabinoid system activation (Pertwee, 2008; Sarne and Mechoulam, 2005). CBD-rich extract showed a linear dose response curve. The dose of CBD necessary to increase the latency for generalized tonic-clonic seizures was bigger than that of THC, and this may be due to different bioavailability of the two extracts, and to different mechanism of action. Many neuroprotective effects of THC are mediated by the CB_1 receptors (Drysdale and Platt, 2003), whereas CBD mechanism of action is still unknown (Mechoulam et al., 2007; Pertwee, 2008). In an *in vitro* model of maximal electroshock both THC and CBD showed good anticonvulsant activity (Wallace et al., 2001); the anticonvulsant effect of THC was blocked by the CB_1 antagonist SR141716A, whereas CBD's activity was not lowered by the antagonist, thus suggesting that a different mechanism of action underlies their anticonvulsant effect. Very few papers have been published about the anticonvulsant action of phytocannabinoids and Cannabis extracts, since the endocannabinoid system has been discovered. Much attention has been devoted to investigations about the neuroprotective effects of endogenous agonists, such as anandamide, and 2-AG, or synthetic agonists such as WIN 55,212-2 and HU-210. In a recent study CB_1 knockout mice and wild-type mice treated

with a CB_1 antagonist showed more pronounced seizures and more severe excitotoxic cell death than untreated mice in a model of kainic acid-induced seizures (Marsicano et al., 2003). Based on the evidence that endocannabinoids production is increased during brain injury and other excitotoxicity models (van der Stelt et al., 2002), endocannabinoids have been proposed as "stout guards" of the CNS (Mechoulam and Lichman, 2003). On the other hand, some of the neuroprotective effects of endocannabinoids and exogenous cannabinoids - like CBD - are not mediated by CB_1 receptors (van der Stelt et al., 2002), thus suggesting the existence of some novel CB receptors (Baker at al, 2006; Pertwee, 2007) or alternative mechanism of action (McHugh et al., 2008). Our preliminary data suggested further investigations about the effect of Cannabis extracts in the PTZ model; at the present, histological evaluations are in progress in our laboratory in order to investigate whether some areas of the brain may be protected from PTZ toxicity in the animals pre-treated with the THC- and CBD-rich extracts. Since at the time of submission of the present thesis histological investigations were still ongoing, we could not conclude whether the effect on latency might lead to neuronal protection in the area involved in the chemoconvulsant activity.

CBD-rich extract was able to increase the survival of the animals to the treatment with a lethal dose of the chemoconvulsant PTZ, and this evidence suggested the possible protective effects in this *in vivo* model. In order to further assess the mechanism of action of CBD, we chose to investigate the effect of CBD in *in vitro* models of excitotoxicity and mitochondrial dysfunction in hippocampal primary cultures, as hippocampus is one of the area of the brain which plays a prominent role in triggering PTZ-induced seizure (Brevard et al., 2006).

Discussion of the in vitro study

Variations in intracellular levels of Ca affect physiological and biochemical processes of the neuron (Berridge et al., 1998; Toescu and Verkhratsky, 2003). In fact, Ca ions are the most important second messengers used for signal transduction (Bootman et al., 2001). The major sources of intracellular Ca are entry into the cell through NMDA receptors or voltage-dependent Ca channels (VDCC), and release from intracellular Ca stores. In physiological conditions, Ca activates a number of Ca-dependent enzymes that influence a wide variety of cellular components, like cytoskeletal proteins or second messengers (Berrigde et al., 2000). Levels of intracellular Ca are regulated by transport proteins that remove it from the cytoplasm; for example, the Na^+/Ca^{2+} exchanger, the endoplasmatic reticulum Ca^{2+} ATPase, and the mitochondrial Ca^{2+} uniporter (Duchen, 2000). Whether directly (ATPase) or indirectly (exchangers depending on electrochemical gradient), these transporters rely on energy to keep working. Maintenance of physiological Ca homeostasis is critical for neuronal activity; too little Ca leads to impairment of normal functioning, too much cytosolic level may lead to neuronal death (Berridge, 1998; Orrenius et al., 2003). The endoplasmic reticulum (ER) is the most investigated intracellular Ca store in the cell; mitochondria are another important storage of this ion. These two organelles seem to be functionally coupled to accomplish Ca buffering (Rizzuto et al., 2004). Less is known about the mechanism of mitochondrial Ca extrusion in neurons, whereas more has been elucidated on the ER mechanisms. Two intracellular Ca release channels are present on ER membrane, the inositol 1,4,5-trisphospate receptor (IP_3R) and the ryanodine receptor (RyR) (Verkhratsky, 2002). The first is activated by IP_3, the latter by cytosolic Ca; since IP_3R is modulated by Ca, they both can be considered Ca gated channels. The size of the mitochondrial Ca pool is smaller than that of ER under physiological conditions (Duchen, 2000). However, mitochondrial Ca uptake plays an important role in regulating Ca signals in the cell, and mitochondria.

Mitochondria and endoplasmic reticulum can co-localize in the cytoplasm, and ER seems responsible for the maintenance of high Ca microdomains close to mitochondria, so that mitochondrial Ca uptake is facilitated (Rizzuto et al., 1993; Rizzuto et al., 1999). Mitochondrial Ca signalling appears to be fundamental in the control of the metabolism of the organelle; for example, increase in matrix Ca concentration in the mitochondria accelerates the enzymatic activities of several dehydrogenases leading to increased NADH levels, and subsequently, to an augmentation of the mitochondrial ATP production (Jouaville et al., 1999). Another crucial factor in regulating and altering the organelle functioning is the balance between production and inactivation of reactive oxygen species (ROS); although mitochondrial ROS generation might constitute an important signalling molecule to modulate cellular signal transduction, under conditions of enzyme substrates overload or Ca overload, the formation of ROS is assumed to play an important role in mitochondrial degeneration/dysfunction (Orrenius et al., 2007; Ott et al., 2007). Hence, as long as Ca buffering is well controlled, both ER and mitochondria are able to maintain the normal physiological activities of the cell. When this balance is altered neuronal death pathways are activated (Verkhratsky and Toescu, 2003). It is known that apoptotic cell death is triggered by extrinsic or intrinsic signalling pathways that induce death-associated proteolytic and/or nucleolytic activities (Taylor et al., 2008); the intrinsic pathway is mitochondria-mediated (Fulda and Debatin, 2006; Dejean et al., 2006; Orrenius et al., 2003). Once damaged, the organelle membrane undergoes permeabilization, and pro-apoptotic messengers, such as cytochrome-c and caspases, are released (Riedl and Salvesen, 2007; Orrenius, 2004). When apoptosis is triggered, a sequence of morphological events leads to nuclear and cytoplasmatic condensation with blebbing of the plasma membrane (Hengartner, 2000); apoptotic bodies are then removed by macrophages or other surrounding cells. Ca-dependent processes are closely connected with caspases activation, and recent findings also indicate that

interfering with the sequestration of Ca into intracellular pools, like the endoplasmic reticulum, can trigger apoptosis as part of cellular stress response (Orrenius et al., 2003; Orrenius, 2007).

Excitotoxicity is the pathological process where the overactivation of glutamate receptors leads to neuronal death, and is thought to play an important role in many neuropathological conditions such as stroke, traumatic brain injury, ischemia, epilepsy (DeLorenzo et al., 2006; Lipton, 1999; Siesjö and Bengtsson, 1989), and neurodegenerative diseases of the central nervous system such as multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and Huntington's disease (LaFerla, 2002; Mattson et al., 2000; Missiaen et al., 2000). The exposure to excitotoxic concentrations of glutamate causes changes in the neuronal physiology; in fact, the excessive and prolonged increase of intracellular Ca is one of the key events for neuronal damage (Deshpande et al., 2007). In addition, the protracted neuronal depolarisation is another sign of the pathophysiology of excitotoxicity. Both Ca excess and depolarisation correlated with neuronal death are triggered by NMDAR activation, but are not counteracted by Ca entry antagonists, when they are used after the insult (Limbrick et al., 2001). Therefore, parallel to efforts for elucidating the mechanisms underlying excitotoxic cascade, discovery of molecules able to dampen these delayed phenomena are currently made for the development of neuroprotective drugs (Chen and Lipton, 2006). In our experimental protocol, we investigated the possible neuroprotective effects of CBD and memantine on hippocampal cultures. In particular, we tried to characterize the effect of these two drugs in a model of excitotoxicity, induced by the administration of a high dose of NMDA using a low Mg^{2+} high Ca^{2+} perfusion solution. Memantine is a non-competitive antagonist of NMDA receptor, approved for the treatment of mild-severe to severe Alzheimer’s disease, and possesses neuroprotective effects (Danysz et al., 2000; Parsons et al., 2007). CBD is one of the most abundant cannabinoid of Cannabis sativa, together with THC. CBD lacks

psychoactivity and has therapeutic potential for the management of inflammation, anxiety, emesis and nausea (Drysdale and Platt, 2003; Platt and Drysdale, 2004); its neuroprotective effects are under an intense investigation over the last decade. Several mechanisms are considered the rational basis of neuroprotective properties of cannabinoids (Drysdale and Platt, 2003): reduction and regulation of transmitter release, modulation of Ca homeostasis and excitability, antioxidant properties, modulation of immune response. CBD seems to accomplish all these requirements, so it seems a promising candidate for neuroprotection; in fact, CBD showed to possess an anticonvulsant activity in animal and in *in vitro* models (Karler et al., 1973; Karler et al., 1974a,b; Wallace et al., 2001); unlike THC, tolerance to CBD activity does not occur, and the mechanism of action does not involve CB_1 receptors. CBD showed neuroprotective effects in *in vivo* models of cerebral ischemic injury (Braida et al., 2003; Hayakawa et al., 2006), and blocked glutamate toxicity in cortical neurons regardless of whether the insult was mediated by NMDA receptors, AMPA receptors, or kainate receptors (Hampson et al., 2000). It is also a good antioxidant agent (Hampson et al., 1998; Hampson et al., 2000; Malfait et al., 2000) and possesses good anti-inflammatory activity (Malfait et al., 2000), probably due to inverse agonism at CB_2 receptor (Thomas et al., 2007). CBD enhances adenosine signalling through inhibition of uptake (Carrier et al., 2006), and increases cerebral blood flow through the serotonergic 5-HT_{1A} receptor (Hayakawa et al., 2006). The mechanism of action of CBD is still unknown, but seems to be related to the endocannabinoid system, though not mediated by CB_1 receptor. Recent findings have shown that CBD reduces striatal atrophy generated by exposure to 3-NP, and that this effect is not mediated by cannabinoid, vanilloid $TRPV_1$ and adenosine A_{2A} receptors (Sagredo et al., 2007), which had been considered putative site of action of CBD.

In our model, the effect of NMDA perfusion was a high rise in the intracellular concentration of Ca within the time of its application; when

NMDA was removed, the $[Ca^{2+}]_I$ dropped to lower levels within five minutes. Since prevention of Ca overloading seems a promising strategy against the excitotoxic insult (Deshpande et al., 2007), we started the investigation with a set of experiments in which CBD and memantine were administered after the insult, to assess their effect on $[Ca^{2+}]_I$; the analysis of the data showed that neither memantine nor CBD applied after the insult could alter the percentage of neurons showing dysregulation and could not aid recovery. Our working hypothesis assumed that protective treatment leads to a higher percentage of neurons that show a full or partial recovery. This choice was based on the finding that, upon NMDA receptors activation by glutamate, there is a one hour window of opportunity for neuroprotection, during which it is possible to reverse the increased of $[Ca^{2+}]_I$ and prevent neuronal death (Deshpande et al., 2007). In the first instance, we assessed that neither CBD nor memantine were able to change the percentage of neurons showing full or partial recovery when perfused after the insult to neurons; in other words, they could not aid neuronal recovery when applied after NMDA. On the other hand, when we analysed the late amplitude parameter, CBD demonstrated the capacity to lower the $[Ca^{2+}]_I$ measured at the end of the washout, suggesting that it had an effect on the mobilisation of Ca after the insult. It is difficult to conclude whether this effect could lead to neuroprotection and improved survival, as further studies are necessary to confirm this. This preliminary result needs to be further investigated: a model of NMDA-induced excitotoxicity and viability study is in progress at the moment in the laboratory. In any case, this data is in agreement with recent findings (Drysdale et al., 2006) that CBD has a role in the intracellular modulation of Ca stores and events secondary to the initial NMDA receptors activation. Memantine neither helped recovery, nor showed any modulation of Ca loading; this is what we could expect from an antagonist of NMDA receptors applied post NMDA application. In fact, as it was recently found, Ca entry

antagonists are not effective in reducing the elevated $[Ca^{2+}]_I$ after excitotoxic insult has occurred (Deshpande et al., 2007).

To further investigate the role of CBD and memantine in neuroprotection, we chose to apply them simultaneously with NMDA. Memantine dampened the NMDA response of the neurons, increased the percentage of neurons showing full recovery, and lowered the $[Ca^{2+}]_I$ measured at the end of the perfusion; on the other hand, CBD applied contemporary to NMDA application did not alter NMDA response, or modify any of the recovery parameters analysed. As it was predictable, memantine, a non-competitive antagonist of NMDA receptors (NMDAR), was able to lower the response of neurons to NMDA; in fact, during the contemporary administration of memantine and NMDA the rise in $[Ca^{2+}]_I$ was strongly dampened. On the other hand, CBD did not influence NMDA response when co-applied; this is in agreement with the findings about CBD's mechanism of action, which does not seem to involve direct interaction with glutamate receptors (Mechoulam et al., 2007). When recovery parameters and late amplitude were evaluated, memantine exhibited a protective effect, as it was able to increase the percentage of neurons showing full recovery; moreover it decreased the $[Ca^{2+}]_I$ measured at the end of the washout. We can conclude that, in this experiment, the delayed excitotoxic events might have not been triggered as memantine prevented the complete activation of the NMDAR; in fact, even if intracellular Ca concentration was very high, the neurons were able to recovery.

When acute brain trauma occurs, the damage to cells does not only involve the area directly affected by lesions. In fact, necrotic cell death is characterized by the irreversible swelling of the cytoplasm and its organelles. Cell lysis, due to loss of membrane integrity, results in the release of noxious cellular constituents, and this leads to inflammation and damage in the surrounding tissue (Dirnagl et al., 1999; Leker and Shohami, 2002). Neuroprotection from this type of insult involve possible enhancement of defensive neuronal and glial mechanism, in order to minimize the spread of

tissue damage (Streit, 2005). Thus, the final investigative path was performed to evaluate the effects of the two compounds when applied before NMDA insult. As previously reported (Drysdale et al., 2006), CBD raised intracellular concentration of Ca when applied to primary hippocampal cultures. Though in the present study we used a different perfusion solution to simulate the excitotoxic insult, the response of CBD seemed to be of similar degree. Here, we used a very high excitability perfusion solution: low concentration of Mg^{2+} was applied to overcome the ion blocking action on NMDA receptors, and high concentration of Ca^{2+} to facilitate Ca overloading inside the cells. Moreover, differently from Drysdale's study, we did not use any channel blocker (like the Na channel blocker TTX): in fact, in order to induce excitotoxicity spontaneous neuronal spiking and Ca oscillations are required. A CBD response was elicited in the experimental condition we used, so we can conclude that its response is independent of Na channel block. In comparison, memantine did not evoke any response when applied to neurons. This is what can be expected, as it is a NMDA receptor antagonist. Differently from what happened when memantine and CBD were applied simultaneously to NMDA (memantine lowered NMDA response, CBD did not), when the drugs were administered before the insult, they both lowered NMDA response. Neither CBD nor memantine were able to improve recovery when applied before the insult, though. Nevertheless, memantine lowered the late amplitude measured at the end of the washout. If this effect of memantine is to be attributed to the decrease of Ca influx during the perfusion of NMDA, it is worth asking why CBD's action on the late amplitude is different from that of memantine. The answer may be that the two drugs have different mechanism of action by which they lower NMDA responses; in fact when they were applied simultaneously to NMDA memantine lowered NMDA response, whereas CBD did not. Most certainly when CBD's mechanism of action will be disclosed, we would be able to further discuss this hypothesis. The Ca imaging results indicate that only

memantine applied contemporary to NMDA is capable of protecting neurons in this model of excitotoxicity, as it is a NMDA receptor antagonist, and prevents total activation of this receptor. Numerous studies have demonstrated that the treatment with NMDA receptor antagonists, during exposure to toxic dosages of glutamate, can prevent further increase of the concentration of intracellular Ca, thus leading to protection of neurons from necrotic death in cortical and hippocampal areas (Coulter et al., 1992; Limbrick et al., 2001); on the other hand, others reported that in conditions of mitochondrial depolarisation or when Ca extrusion/sequestration mechanisms are impaired, blocking NMDA receptor does not prevent further neuronal damage (Limbrick et al., 2001; Norris et al., 2006; Vergun et al., 1999). So, when the mechanisms which regulate Ca homeostasis in the cells are already damaged, neurons are more susceptible to delayed excitotoxicity, whereas there is a time window when neurons can be effectively protected from further insult by dampening NMDAR-induced Ca currents (Deshpande et al., 2007). Here, we used young hippocampal cultures, and showed how memantine applied simultaneously to NMDA helped recovery from the insult, whereas pre- or post-application were ineffective in restoring normal levels of Ca.

The possible neuroprotective activity of CBD and memantine was also investigated in a model of impairment of cell energetic metabolism, using the mitochondrial toxin 3-nitropropionic acid (3-NP). 3-NP inhibits succinate dehydrogenase in the tricarboxylix acid cycle, and alters energy dependent processes in cells, as this enzyme is necessary for the synthesis of ATP by mitochondria (Alston et al., 1977; Coles et al., 1979). 3-NP induced neuronal loss is shown to be mediated by excessive activation of glutamate receptors, leading to the production of hydroxyl radical and peroxynitrite (Beal et al., 1993). In particular, energy deficiencies may cause cellular depolarisation, and NMDA receptor activation, and the Ca cascade may increase the damages to cell. That explains why 3NP toxicity has been reported to be

attenuated by glucose (Fink et al., 1996). In this study, memantine showed neuroprotection, as it was able to increase the number of viable neurons in the intoxicated dishes. Neuronal and glial function is critically dependent on the maintenance of electrochemical gradients across membranes, and around 60% of ATP produced by the cell is necessary to the Na^+/K^+ ATPase working (Hansen, 1985). Consequently, when energy production is impaired (i.e. during hypoxia), a rapid loss in ionic homeostasis occurs, and the cell depolarizes. NMDA receptors and voltage-dependent Ca channels can be activated, and the intracellular Ca stores may fail to accomplish Ca buffering. When the Ca homeostasis of the cell is altered, excessive entry of Ca initiates a series of cytoplasmatic and nuclear processes that promote neuronal cell death: activation of proteolytic enzymes, activation of Ca^{2+}/calmodulin kinase II (with increased activity of phosphorylated enzymes), activation of Ca-dependent endonucleases, and expression of transcription factors (Berridge et al., 1998; Orrenius et al., 2003). Here, memantine was able to decrease the Ca entry into neurons, as it blocks NMDA receptors as soon as they are activated by loss of the membrane potential. This mechanism of action can explain the protective effect in our model of energy impairment; this finding is in agreement with the neuroprotective effect of another non-competitive NMDAR antagonist, MK-801, in models of metabolic impairment (Zeevalk et al., 1995). Previous studies have shown that glial cells can be damaged by 3-NP, but at higher doses than that used in the present study (Ryu et al., 2003). On the other hand, CBD did not show any protective effect in this model. 3-NP toxicity is mediated by oxidative stress and ATP depletion (Alston et al., 1977; Coles et al., 1979). It was surprising that CBD did not show protection in this model, because its neuroprotective effect and that of other cannabinoids is assumed to involve their antioxidant capacity (Drysdale and Platt, 2003; Hampson et al., 1998; Marsicano et al., 2002). Since only memantine was able to protect neurons from 3-NP toxicity, we can argue that targeting NMDA receptor may exert protection in this model in hippocampal

cultures. Such evidence is in agreement with the report that acute 3-NP toxicity is glutamate-receptor mediated in striatal, hippocampal, and hypotalamic neurons (Fink et al., 1996; Pang and Geddes, 1997), and that NMDA antagonist, MK-801 can attenuate 3-NP-induced necrotic death (Rajdev and Reynolds, 1994). It is worth mentioning that a very recent paper (Sagredo et al., 2007) showed that CBD may act as neuroprotective agent, by reducing the striatal atrophy generated by in vivo exposure to 3-NP. As others before (Hampson et al., 2000; Wallace et al., 2001), they demonstrated that CBD neuroprotection was independent from CB_1 receptors interaction. The lack of activity in our 3-NP intoxication model could be attributed to the different types of neurons studied, and to differences between *in vivo* and *in vitro* experiments.

Altogether, our data showed that the NMDA antagonist memantine successfully protected hippocampal neurons from excessive loading of Ca, and prevented damage to cellular structures. Memantine clearly showed its mechanism of action on the NMDA receptor in the Ca imaging experiments, leading to a decrease of Ca influx. This decrease in Ca influx might also explain the protection shown in the viability study. In contrast, CBD did not provide major protection in both models, but was able to reduce NMDA response and secondary events, leading to a significant decrease of Ca inside the neurons. This effect is indicative of intracellular sides of actions subsequent to the activation of the Ca cascade.

CONCLUSIONS

In the present thesis we investigated the possible effect of CBD in models of convulsion and excitotoxicity. In the *in vivo* study CBD was administered as the major cannabinoid of a Cannabis extract, and showed the capacity to increase the latency to chemical-induced convulsions in mice, and to increase the survival to the treatment with a lethal dose of the chemoconvulsant PTZ. In the *in vitro* study we investigated the possible neuroprotective effects of pure CBD in models of excitotoxicity and mitochondrial impairment in hippocampal cultures, and compared its activity to that of the NMDA antagonist memantine. Although CBD did not show major protection in either models relative to memantine, it showed the capacity to modulate intracellular Ca levels. This effect is to be taken into great consideration as maintaining proper Ca homeostasis is critical for the viability of neurons (Berridge et al., 2000; Toescue and Verkhratsky 2003), and evidence is accumulating about the fact that perturbation in Ca homeostasis is the main cause of neuronal loss, in chronic and acute neuropathological conditions such as Alzheimer's, Parkinson's, and Huntington's diseases, stroke, and epilepsy (DeLorenzo et al., 2006; LaFerla, 2002; Lipton, 1999; Mattson et al., 2000; Missiaen et al., 2000; Siesjö and Bengtsson, 1989). CBD's mechanism of action and neuroprotective effects have been under intense investigation over the last decade, and as this compound lacks psychoactivity and is tolerated well in humans (Pertwee, 2004), it seems a good candidate for possible future clinical applications. Overall, the present investigation supports CBD's role in neuroprotection and calls for further studies beyond the preliminary results obtained here.

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