DRUG ABUSE, BRAIN CALCIFICATION AND GLUTAMATE-INDUCED NEURODEGENERATION

MJ Rodríguez, M Pugliese and *N Mahy

Unitat de Bioquímica, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Facultat de Medicina, Universitat de Barcelona, Barcelona, Spain.

Corresponding Autor:

Dr Nicole Mahy,

Unitat de Bioquímica, IDIBAPS, Facultat de Medicina, Universitat de Barcelona

c/Casanova 143, 08036, Barcelona, Spain

Tel. +34 934024525

Fax +34 934035882

e-mail: nmahy@ub.edu

LIST OF ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid;

ATP: Adenosine triphosphate

$[Ca^{2+}]_i$: Intracellular calcium concentration;

CNS: Central Nervous System;

GABA: γ-aminobutyric acid;

GluR: Glutamate receptor;

K_{ATP} channels: ATP-dependent potassium channels

mGluR: Metabotropic glutamate receptor;

NMDA: N-methyl-D-aspartate;

NR: NMDA receptor subunit:

ROS: Reactive oxygen species;

SK channels: Ca^{2+}-activated potassium channels of small conductance

VTA: Ventral tegmental area;

ABSTRACT

Positive and negative reinforcing systems are part of the mechanism of drug dependence. Drugs with abuse potential may change the manner of response to negative emotional stimuli, activate positive emotional reactions and possess primary reinforcing properties. Catecholaminergic and peptidergic processes are of importance in these mechanisms. Current research needs to understand the types of adaptations that underlie the particularly long-lived aspects of addiction. Presently, glutamate is candidate to play a role in the enduring effects of drugs of abuse. For example, it participates in the chronic pathological changes of corticostriatal terminals produced by methamphetamine. At the synaptic level, a link between over-activation of glutamate receptors, $[Ca^{2+}]_i$ increase and neuronal damage has been clearly established leading to neurodegeneration. Thus, neurodegeneration can start after an acute over-stimulation whose immediate effects depend on a diversity of calcium-activated mechanisms. If sufficient, the initial insult results in calcification and activation of a chronic on-going process with a progressive loss of neurons. At present, long-term effects of drug dependence underlie an excitotoxicity process linked to a polysynaptic pathway that dynamically regulates synaptic glutamate. Retaliatory mechanisms include energy capability of the neurons, inhibitory systems and cytoplasmic calcium precipitation as part of the neuron-glia interactions. This paper presents an integrated view of these molecular and cellular mechanisms to help understand their relationship and interdependence in a chronic pathological process that suggest new targets for therapeutic intervention.

KEYWORDS: Calcium precipitation, Central Nervous System; Addiction; Drug abuse; Excitotoxicity; Glutamate; Neurodegeneration.

INTRODUCTION

Long-term neurological effects are inherent to acute and chronic drug abuse and the complex phenomenon that results from interaction between a variety of biologic and molecular factors remains difficult to address. This is in part due to the absence of clear, long-term markers of drug use and to the limited models that investigate the whole process of drug abuse and addiction in specific brain areas. Besides none of these models reproduces all the human features, the usefulness of each one depends in large part on its validity as simulation of human behaviour. Many acute and chronic animal models associated with different paradigms are aimed at the determination of the involvement of neurotransmitters like serotonin, dopamine, noradrenaline, endo-cannabinoids, γ-aminobutyric acid (GABA) or glutamate in several brain areas, at the investigation of specific aspects of the drug effects or at the analysis of the drug addiction process, The behavioural paradigms of euphoria and rewarding effects, including self-stimulation, self-administration, and conditioned place-preference models, are mostly used for the identification of the neuronal pathways involved in addiction [1;2]. All addictive drugs facilitate dopamine transmission and produce alterations in other neurotransmitters. For example, repeated cocaine produces in the rat a marked dopamine and glutamate release in nucleus accumbens and other areas such as ventral tegmental area and striatum [3] and ectasy abuse results in increased serotonin, dopamine and noradrenaline release [4]. The animal model with self-administration by various routes (e.g., oral, intragastric, intravenous, intracranial) is still the gold standard for assessing the rewarding properties of drug abuse and is commonly used for the preclinical assessment of the abuse liability of new agents [5]. In this model, experimental animals, normally a rat or a monkey are tested in a chamber containing a lever. Animals are trained to perform an operant task and drug delivery is made

contingent on the performance of an operant response, typically lever pressing or nose poking. With this technique, normal animal behaviour (e.g., grooming, feeding and drinking) can be studied concurrently with drug self-administration, but not the complexity of the molecular events.

In recent years knockout mice have helped identify the proteins mostly in charge of a specific response or behaviour [6]. Mice lacking a specific receptor, transporter or enzyme are used to determine whether this molecule plays a key role in the acute response or the addiction process [7]. For example, mice lacking the μ receptor neither exhibit the behavioural effects nor become physically dependent when given opioids. Reverse genetic approaches is also applied to identify the underlying molecular components. Thus, models lacking a dopamine transporter, noradrenaline transporter or vesicular monoamine transporter and exposed to psychostimulants, tricyclic antidepressants or reserpine present an enhanced behavioural response explained by the direct or indirect dopamine receptor activation

Alcoholism, an example of polygenic disorder, depends on gene-environment interactions [8]. Its chronic ingestion alters multiple pathways and modifies the serotonin, nicotinic, γ-aminobutyric $(GABA)_A$, N-methyl-D-aspartate (NMDA) and μ and δ receptors. The absence of alcohol self-administration behaviour in μ receptor-k.o mice has been determinant to strongly involve this receptor and clarify, in part, the addiction process.

The ventral tegmental area (VTA) is necessary for the processing of rewarding of drugs of abuse, where they rapidly potentiate excitatory synapses on dopaminergic neurons, and alter GABAergic synapses. However, whereas the nature of the main pathway initially involved, the function of proteins that regulate pre- and postsynaptic glutamate neurotransmission appears modified in several brain areas. To illustrate the key

participation of glutamatergic transmission in circuits involved in drug abuse, cocaine, amphetamine and ethanol molecular actions are considered in more details.

GLUTAMATE NEUROTRANSMISSION, REWARD CIRCUIT AND DRUGS OF ABUSE

The involvement of interconnected circuits between prefrontal cortex, hippocampus, nucleus accumbens, VTA, and amygdala in the development of addiction and in the manifestation of addictive behaviours implies the participation of many neurotransmitters. These organized circuits have GABAergic, peptidergic and glutamateric outputs, and activation of glutamatergic synapses seems critical in the expression of addictive behaviours. Glutamatergic transmission accounts for up to 70% in the Central Nervous System (CNS), and there are glutamatergic projections and/or neurons expressing glutamate receptors in most circuitries, including the reward one. Thus, VTA and its different targeted regions, like nucleus accumbens, amygdaloid complex and frontal cortex receive substantial glutamatergic input [9]. Modification of glutamatergic function, a mechanism central to neuronal plasticity, is involved in short-term and long-term drug effects. In this way, activation of glutamatergic pathways is a critical point in the development of addictive behaviours for a variety of drugs including, cocaine, heroin, nicotine and alcohol. As such, glutamate mediates numerous effects of acute and chronic exposure to cocaine. For example, metabotropic glutamate receptor 5 that has the potential to directly regulate neurons is essential for cocaine self-administration and behaviour [10]. Acute administration induces a redistribution of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and NMDA receptors in the VTA and a reduction of various ionotropic glutamate receptor (GluR) subunits expression, like the GluR3, GluR4 and NMDA receptor subunit 1 (NR1) in the nucleus

accumbens. This may be viewed as a compensatory mechanism of the increased synaptic glutamate caused by cocaine, especially after high doses (30mg/kg, i.p.) [11]. Chronic administration results in behavioural sensitization to cocaine, with an increased responsiveness of the dopamine mesolimbic system to glutamate due to a specific increased responsiveness of AMPA receptors and an increased expression of GluR1 of the VTA [12;13]. Some authors have also found in this area a specific increase of NR1 [14;15]. Because of the numerous procedural differences, changes in other GluR subunit expression as an adaptative response to repeated cocaine administration or withdrawal are not conclusive [16;17]. Because modifications are only detected associated with behavioural signs of sensitization; lack of data in other circumstances may overlook subtle changes, such as an increased surface expression of AMPA receptor subunits that remain to be clearly established [18;19]. In addition, involvement of glutamatergic transmission in cocaine reward, reinforcement and reinstatement has been clearly established by direct glutamatergic fibber stimulation or blockade of NMDA and AMPA VTA receptors [20;21]. However, how each component of the glutamatergic synapse participates and adapts to cocaine repeated exposure remains to be deciphered. Research with genetically modified mice currently in process may be the better choice to clarify the role of each proposed target. Preliminary results indicate a preponderant role of the Homer proteins in the behavioural sensitizing effects [22;23]. These proteins that link NMDA and metabotropic glutamate receptors (mGluR) with intracellular calcium stores might be reduced in cocaine reward behaviour.

As for cocaine, increased overflow of glutamate is associated with amphetamine exposure [24;25]. In this condition, modifications of AMPA and mGlu receptor conductance underlie the well-known increased expression of c-fos and Zif268, and phosphorylation of transcription factors [26;27]. mGluR also mediate VTA neuron

plasticity induced by amphetamine [28]. Glutamatergic neurotransmission has been correlated with the neurotoxic effects of high doses of amphetamine [29]. However, the marked differences in glutamatergic neurotransmission caused by acute and chronic amphetamine exposures rise several questions related the plasticity of the glutamatergic synapse [30-32]. These questions mostly concern how modification of neuronal responsiveness takes place, how reduction in NMDA receptor components is accompanied by short and long-lasting modifications in AMPA and mGluR receptors, and how astroglia participates of these modifications. Finally, more research is also needed to better understand the role of glutamate in the reinforcement and relapse-behaviour of amphetamine exposure.

Research on glutamatergic neurotransmission and ethanol abuse is present from the late 80-90. Before, ethanol increased membrane fluidity and potentation of GABAergic neurotransmission were considered as its two basic mechanisms of action. Presently, ethanol inhibition of NMDA receptors resulting in an up-regulation of various NMDA receptor subunits in a chronic ingest has been well established [33-35]. Thus, consistently, several NMDA receptor subunits (NR1, NR2A and NR2B) are increased in VTA, amygdala, cerebral cortex and hippocampus and support an increased conductance and cationic influx [36-38]. However, ethanol effects on glutamate overflow depends on its concentration: at low dose synaptic glutamate levels are increased, whereas they are reduced at high ethanol doses [39;40]. Astroglia has been suggested to participate in this paradoxical effect, but research is needed to clearly understand the underlying cellular and molecular mechanisms [41]. The same is true for the effects of withdrawal. Acute manifestations include increased synaptic glutamate and hyper-excitability whose intensity can reach seizure-like level [42;43]. One of the main questions to be presently answered by researchers is why these manifestations

increase in intensity after repeated withdrawal periods. This question relates with the elevated risk factor of cerebrovascular diseases such as stroke present in ethanol abuse [44;45]. Finally, exposure to ethanol during synaptogenesis of the developing CNS causes an important neuronal loss due to the pro-apoptotic effect of ethanol. As a result, the disruption of synaptic connections and massive neuronal death explain the diminished brain size and disturbances associated with the foetal alcohol syndrome. In rodents, this neuronal loss only requires a single low ethanol intoxication episode (80mg/dl-60min) to trigger neurodegeneration [46;47]. Besides its major interest, research of long-term effects of drug exposure on CNS maturation is presently limited by the available experimental models and required time of study.

EXCITOTOXICITY AND NEURODEGENERATION

In the early 1970s, Olney defined excitotoxicity as the neuropathological process triggered after over stimulation of excitatory amino acid receptors [48]. At present, excitotoxicity includes the concept of glutamate-mediated endogenous neurotoxicity; i.e. the putative excitotoxicity when glutamate increases in the extracellular space [49]. This concept is of interest because it presents the possibility of new strategies in pharmacological neuroprotection. It is generally accepted that excitotoxic injury to neurons results from excessive inward currents of Ca^{2+} and Na^{+} through glutamate-operated ion channels -i.e. kainate, NMDA, or AMPA receptors-, supplemented by release of Ca^{2+} from intracellular stores subsequent to mGluR activation, leading to intracellular Ca^{2+} overload [50]. Excitotoxicity also involves an imbalance of transmembrane Na^{+}, Cl^{-} and K^{+} gradients, cell swelling [51] and formation of calcium precipitates in most CNS areas [52-54]. The complexity of the mechanisms involved in glutamatergic neurotransmission makes it already apparent that a number of

abnormalities, pre-synaptic, post-synaptic or glial, alone or in combination can be excitotoxic. For example, a loss of selectivity of ionotropic receptors [49], or deficiencies in glial re-uptake of glutamate [55] are observed in lateral amyotrophic sclerosis. In drug abuse, synaptic glutamate dysfunctions contribute to explain phenomena such as the effectiveness of either a mGluR1, mGluR5 or NMDA antagonist to inhibit the up regulation of endoplasmic reticulum stress protein due to acute and chronic cocaine administration [3].

Excessive glutamate-mediated excitation stands out as a critical factor common to neurodegeneration [49;50;56]. Neurodegeneration can start after an acute injury whose immediate effects depend on a diversity of Ca^{2+} activated injuring and compensatory mechanisms. For example, opioid blockade of long-term potentiation of $GABA_A$-mediated transmission onto dopamine neurons in the VTA potentiates excitatory synapses [57], and may activate a chronic on-going neurodegenerative process. This sustained over-stimulation of glutamatergic synapses also observed after high doses of methamphetamine results in an excitotoxic process with a decrease in NMDA receptor binding and dopamine striatal content [58]. In fact, long-term dopamine toxicity associated with methamphetamine abuse is directly caused by glutamate receptor overstimualtion [59].

A link between intracellular calcium concentration ($[Ca^{2+}]_i$) increase, over-activation of excitatory amino acid receptors, and neuronal death has been established from data obtained in *in vitro* and *in vivo* models [60-62]. In animal models, excitotoxicity can be reproduced with low doses of glutamate agonist microinjections. Due to the high affinity of ionotropic glutamate receptors for specific agonists, NMDA, AMPA or kainate injected in non saturable conditions are able to trigger calcium-mediated

excitotoxicity in several rat brain areas and an on-going neurodegenerative process [63].

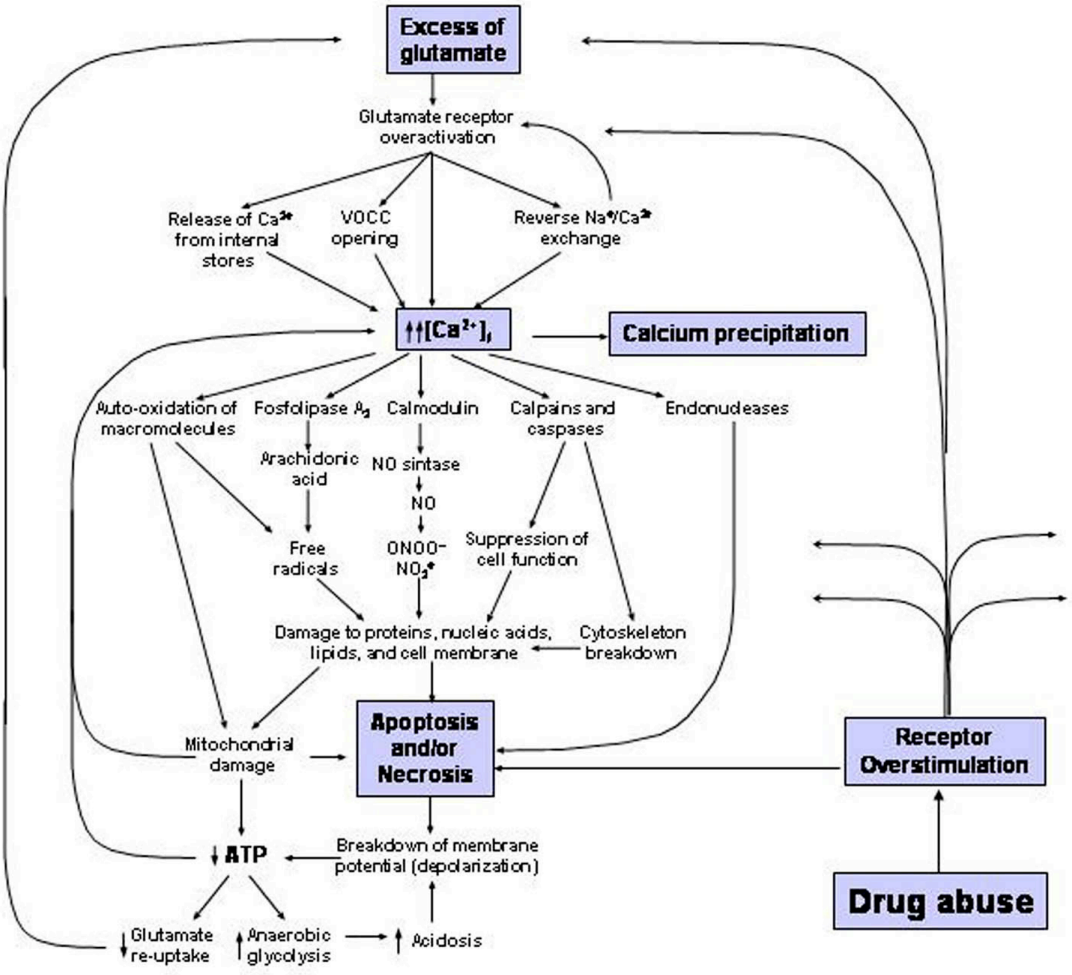

Fig. 1: Cellular and molecular alterations induced by drug abuse. Drug abuse implies overactivation of several pathways, including the glutamatergic one. The overstimulation of glutamate receptors results in an uncontrolled increase of $[Ca^{2+}]i$ and apoptotic or necrotic neuronal death. Positive feedback of the lesion induces glutamate release during the whole process (Adapted from [62]).

Calcium overloading in cytosol and mitochondria plays a critical role in neuronal injury (Fig 1.), but the superfluous Ca^{2+} also induces release of several intrinsic and extrinsic factors that activate processes to rescue neurons from death [64-67]. As a result, and to support their low regenerative capacity and long life span, neurons may withstand very

large calcium insults through adaptative mechanisms. For example, in presence of excess phosphate, the formation of calcium phosphate complexes sequesters free calcium in neurons and astrocytes at no energy expense [68-71]. Dynamic changes in calcium movements after drug abuse are mainly associated with over-stimulation of dopamine and glutamate post-synaptic receptors leading to oxidative stress and toxic effects [72]. Thus, since cocaine increases levels of glutamate and dopamine, further $[Ca^{2+}]_i$ increase depends directly o the graded receptor activation and on the participation of adaptive mechanisms.

Brain calcification represents a new adaptative step of calcium homeostasis

Glutamate receptor agonist microinjection in rat CNS mimics the intracellular Ca^{2+} precipitation present in human and canine degenerative process [52;63;73]. Similar calcification is also observed after blockade of synaptic glutamate re-uptake [55]. As these deposits are observed in several areas of rat brain after microinjection of different excitotoxins [63;71;73;74], their formation does not depend on the glutamate receptor subtype initially stimulated. However, their size, number and distribution vary with both the activated receptor and the CNS area. Ca^{2+} deposits do not occur in all cells that degenerate in response to excitotoxins. For example, in the basal forebrain and medial septum, the calcification observed in GABAergic cells was not detected in cholinergic neurons. The former, together with astrocytes, seem to participate actively in the calcification process [52;75]. X-ray microanalysis showed an electron-diffraction ring pattern which was characteristic of a crystalline structure similar to apatites [76], and a Ca/P ratio of 1.3±0.2 of cytoplasmic deposits (fig. 2), a ratio lower than the theoretical apatite value of 1.67. This ratio is also typical of biological crystals which do not have an ideal organization [71]. As biological hydroxyapatites, these deposits are similar to

those observed in several peripheral human tissues [77;78]. Therefore, calcification depends on the increase in intracellular inorganic phosphate (i.e. adenosine triphosphate (ATP) depletion) and, most importantly, on the degree of protein phosphorylation. Thus, the Ca^{2+}-binding-protein-dependent kinases and the activity of neurotrophic factor ultimately control calcification.

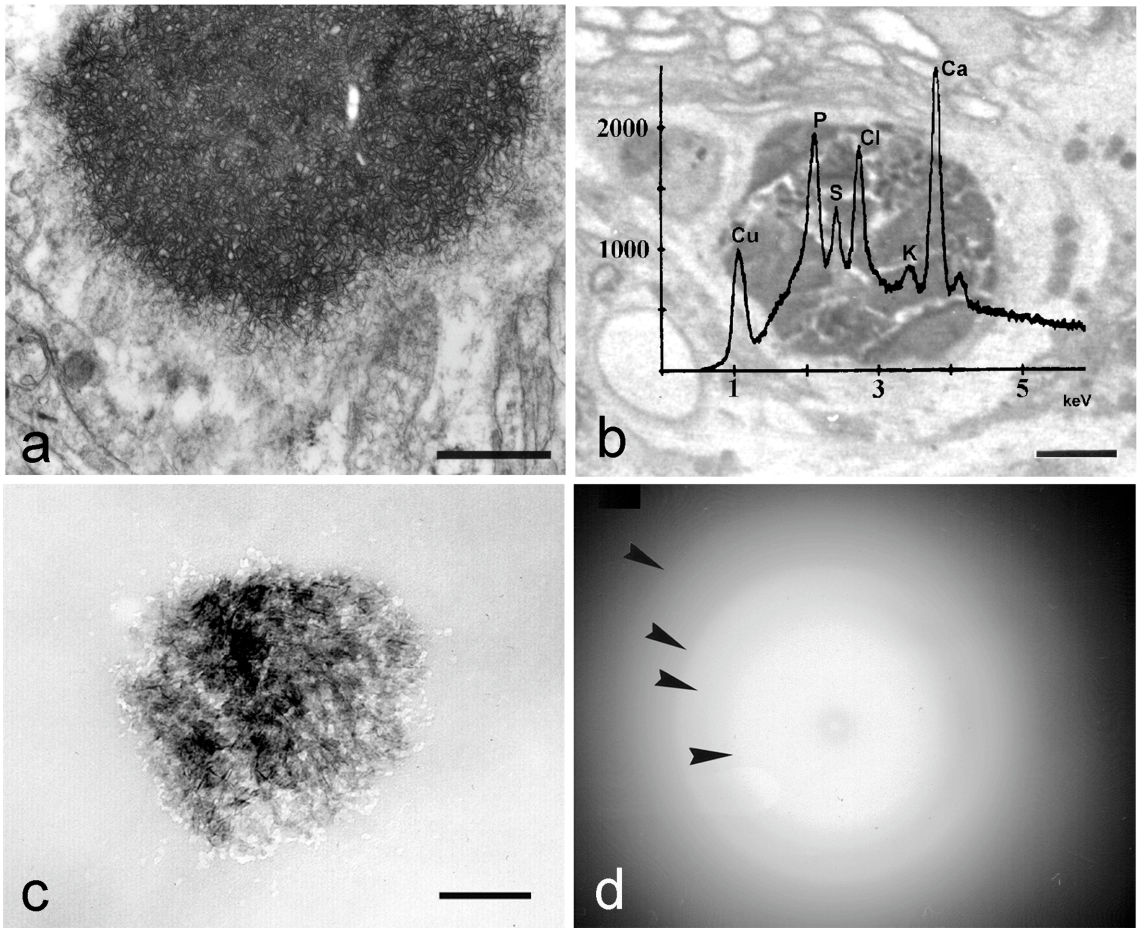

Fig. 2: Characterization of Ca^{2+} deposits. a) Intracellular Ca^{2+} viewed by TEM; note its acicular structure composed by several nanocrystals. b) X-ray microanalysis of a non-osmificated brain sample with a calculated Ca/P ratio of 1.3. c) TEM image of a non-osmificated deposit showing needle-shaped crystals. d) Electron-diffraction image with a four-ring pattern (arrowheads) similar to that of hydroxiapatite. Bars: a, 0.5 μm; b, 5 μm; c, 0.2 μm.

Together with Ca^{2+} deposits, glutamate ionotropic receptor over-stimulation induces precipitation of uric acid and aluminosilicates, and the accumulation of sulphated mucosubstances [75]. The formation of these products may be related to the appearance of tissue compensatory mechanisms. Uric acid, the end product of adenosine and guanosine catabolism, increases after nucleic acid degradation, acts as antioxidant and protects mitochondria against glutamate-induced $[Ca^{2+}]_i$ increase [79]. Moreover, adenosine inhibits neurotransmitter release and a balance between excitatory and inhibitory neurotransmission may prevent glutamate excitotoxicity [80;81]. Consequently, the concentration of uric acid increases during neurodegeneration [82] and, due to its limited solubility in physiological conditions, it easily precipitates as urate crystals. Crystallization of aluminosilicates may also be related to a compensatory mechanism of $[Ca^{2+}]$i increase [83] because of the unique affinity of aluminium for silica acid. Precipitates of hydroxyaluminosilicates are therefore easily formed to reduce aluminium toxicity. Similar cerebral formations have been described in several pathologies such as Alzheimer's or Fahr's diseases, where they would have a similar role. The functional meaning of mucosubstance accumulation remains unclear. *In vitro* mineralization models indicate that glycosaminoglycans and proteoglycans are effective competitive inhibitors of hydroxiapatite formation and growth [84]. This suggests that their accumulation in brain may reduce $[Ca^{2+}]_i$ through Ca^{2+} sequestration. However, if phosphorylated, they may participate directly in the nucleation of hydroxiapatite formation [84]. It should also be noted that, because of their high sulphur content, these mucosubstances may act as antioxidants

The $[Ca^{2+}]_i$ increment finally activates the mechanisms triggering neuronal death, Ca^{2+} extrusion and buffering are activated when the $[Ca^{2+}]_i$ increases [62;85] with a great expenditure of energy through Ca^{2+}-ATPases. The replacement of damaged molecules

also depends on ATP availability. Moreover, the high mitochondrial intake of Ca^{2+} can lead to a loss of the mitochondrial membrane potential and the production of reactive oxygen species (ROS), thereby decreasing cellular respiratory capacity. As a result, aerobic glycolysis accelerates during the period soon after acute excitotoxicity; however, because of the limited mitochondrial function, pyruvate is transformed into lactate with the only gain of 2 ATPs per molecule of glucose. Therefore limited ATP forces a reduction in astroglial energetic consumption to facilitate neuronal glucose availability [86] and helps maintain neuronal membrane polarity as a priority. In this situation, intracellular Ca^{2+} may precipitate as hydroxiapatite to reduce its cytoplasmic toxicity as well as the extrusion energy expenses in neurons and astrocytes.

The massive astroglial production of lactate to help compensate neuronal energy depletion caused by excitotoxicity is a key factor in brain calcification. pH reduction associated with increased lactate concentration facilitates the solubility of Ca^{2+} and the formation of $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-} ions from inorganic phosphate and phosphorylated proteins. Because of the very high Ca^{2+} / $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-} affinity, apatite nucleation may occur with the subsequent growth of crystalline formation along with neurodegeneration (Fig 3.). If this is the case, calcification of each lesioned area depends not only on the density and subtype of glutamate receptors, phosphate availability and Ca^{2+} movements, but also on the differential capacity of glial cells to release lactate during degeneration. These concretions are intimately associated with mucopolysaccharides acting as templates that also help neutralize in the injured cell reactive oxygen species [75;87].

Evidence has been provided of a common pattern of brain calcification taking place in several human pathologies, and in the rat with glutamate-derived CNS lesions, regarding the chemical composition, physical characteristics, and histological

environment of the precipitates. Furthermore, a common physical mechanism of deposit formation through nucleation, lineal growth, and aggregation has been proposed, under the modulation of protein deposition and elemental composition factors [70]. Insofar as calcium precipitation reduces activity signals at no energy expense, the presence in human canine and rodent brain damage of a common pattern of calcification may reflect an imbalance between cellular signals of activity and energy availability for its execution.

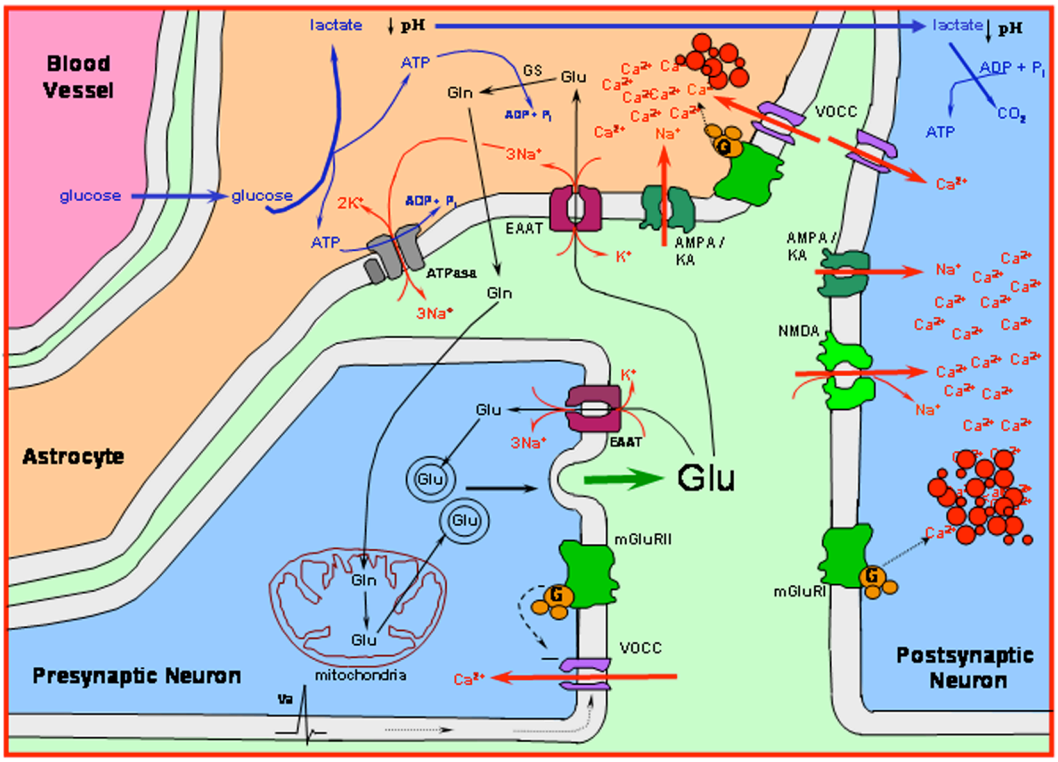

Fig. 3: Schematic drawing of the excitotoxic process induced by glutamate with calcium precipitation as an adaptative new step of Ca^{2+} homeostasis. The massive astroglial production of lactate that intends to adapt to the increased neuronal energy requirement caused by excitotoxicity is a key factor for brain calcification. pH reduction associated with increased lactate concentration facilitates the solubility of ions from inorganic phosphate and phosphorilated proteins. Because of the very high Ca^{2+} /

$H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}, affinity, apatite nucleation may easily occur with the subsequent growth of crystalline formation along with neurodegeneration.

Finally, the lack of brain calcification long time after injection of low doses of excitotoxin qualifies calcification as an acute process. Due to this correlation established with acute but not chronic brain damage, the extension of calcification in a brain area depends on the intensity of the acute phase of each pathology [70;88]. Thus, the positive correlation between the calcified area of hippocampal formation and extension of damage found in blood flow neuropathies such as hypoxia-ischemia and vascular dementia [89], is not found in Alzheimer's disease [70;88]. Calcium deposits present within the amyloid plaques of Alzheimer's patients would reflect the compensatory mechanisms activated by the same plaque toxicity. To our knowledge, until now, no study has been done to assess the presence of brain calcification in drug addicts. If present it would reflect the cumulative effects of repeated acute damage rather than the chronic lesion. As said earlier, this would be expected in the periods of repeated withdrawal of chronic alcoholism, associated with an hyperactivity of the glutamatergic synapse. If true, calcification detection by brain imaging could be of major interest because of its strong correlation with neuronal death, as evidenced in perinatal human hypoxia-ischemia [88].

Overstimulation, intracellular calcium increase and energy failure

The increase in $[Ca^{2+}]_i$ and the energetic loss can induce other interdependent mechanisms that underlie neuronal death, such as acidosis, ROS generation, and activation of proteases and endonucleases that trigger apoptotic death.

Excitotoxicity induces acidosis in cells and in the extracellular space [90]. There are several mechanisms by which pH decreases during neuronal injury. Mitochondrial damage forces the cell to a shift from aerobic to anaerobic metabolism; as a result lactate is produced with the formation of two ATPs and the release of two protons. After trauma and ischemia, extracellular lactate increases dramatically and the pH decreases. To ensure neuronal viability during and even after human hypoxia, glial glucose is oxidized only to lactate, which is rapidly transported into neurons for its complete oxidation [91]. Furthermore, H^+ also appears during some chemical reactions such as phospholipid hydrolysis. In parallel, Ca^{2+} influx causes rapid cytoplasmic acidification through a) the activity of membrane Na^+/H^+ exchanger to restore the Na^+ gradient, and b) the Ca^{2+}-dependent displacement of protons bound to cytoplasmic anions [62].

Oxidative stress produced by methamphetamine is reflected by increases in lipid peroxidation, oxidized proteins in striatum and hippocampus, and reduction in striatal glutathione [92;93]. These toxic effects are the direct result of over-stimulation of GluRs, with the resultant activation of calcium-activated proteins. Agents that protect glutamate overflow protect from subsequent striatal toxicity, despite increases in synaptic dopamine [59]. High $[Ca^{2+}]_i$ derived from drug acute effects can activate a Ca^{2+}-dependent protease which catalyzes the xantine dehydrogenase conversion to xantine oxidase. It also can induce ATP degradation to hypoxantine, a substrate of xantine oxidase together with O_2 the other substrate of the reaction. Consequently, xantine oxidase is strongly activated and produces large amounts of uric acid to prevent further oxidative damage. However, due to its limited solubility, uric acid may precipitate and thus participate to neuronal suffering [82].

Protease activation, apoptosis and necrosis

Activation of proteases of the caspase and calpain families can be triggered by Ca^{2+} influx and oxidative stress. Ca^{2+} overload also activates endonucleases, a series of Ca^{2+}-dependent enzymes that degrade DNA and that may be involved in two morphologically distinct forms of neuronal degeneration: necrosis and apoptosis [62]. Necrosis is a chaotic process that involves rapid energy loss, acute swelling, and vacuolation of the cell body and neurites with subsequent lysis of the cell, which spills the cells contents into the extracellular fluid. Apoptosis involves protein synthesis, compaction of the cell body, nuclear fragmentation, and formation of cell surface blebs that may prevent exposure of surrounding cells to the content of the dying cell [94]. The dysregulation of neuronal Ca^{2+} homeostasis during acute insults may result in excessive stimulation of calpains. Concerning caspases, there are at least two major pathways by which the initiator pro-caspases are activated in response to death-inducing stimuli and subsequently cleave the effector enzymes. Calpain is activated in most forms of necrosis and in some forms of apoptosis, while caspase 3 is only activated in neuronal apoptosis [95]. Calpains could become over-activated under extreme conditions that result in sustained elevation of cytosolic Ca^{2+} levels, which is generally associated with necrosis. Caspases, like calpains, are cytosolic cystein proteases, but do not require Ca^{2+} for activity [95], although they are also responsive to increase intracellular concentration of this ion. Calpains and caspases have a finite number of cellular proteins as substrates, including cytoskeletal proteins, enzymes involved in signal transduction, cell-cycle proteins, and nuclear-repairing proteins. Interestingly, NMDA and AMPA receptors also appear to be substrates for calpains and caspases. Collectively, these findings suggest key roles for caspases and calpains in modulating neuronal Ca^{2+} homeostasis and in preventing excitotoxic necrosis [96]. Additional calpain and caspase substrates

that may be involved in regulating plasticity have been identified in studies of two proteins linked to Alzheimer's disease: β-amyloid precursor protein and presenilin-1. In addition to these two molecules, several other proteins linked to neurodegenerative disorders, such as amyotrophic lateral sclerosis and Parkinson's disease, are caspase substrates.

Although it was initially accepted that excitotoxicity leads to necrotic death, a wide continuous spectrum of situations between apoptosis and necrosis has been described [97]. The factors that determine the pattern of neuronal death seem to be the intensity of the lesion, the $[Ca^{2+}]_i$ and the cellular energy capacity [98]; the apoptotic death is associated with a combination of all factors that results in a less severe injury. The cell then prevents the uncontrolled release of intracellular compounds (e.g. glutamate) and the subsequent inflammatory response of tissue. As ATP levels decrease, the necrotic process starts presenting a hybrid pattern of both neuronal deaths.

RETALIATORY MECHANISMS AGAINST ACUTE SYNAPTIC OVERSTIMULATION

Given these toxic effects, adaptations that act to control glutamatergic neurotransmission and calcium movements in the cell can potentially be protective. At a time scale, these defenses are developed to act at any moment during the excitotoxic event, involve different cellular types such as neurons, astrocytes and microglia, and deal with the cellular and molecular mechanisms of glutamatergic neurotransmission. These mechanisms include defenses that: a) decrease neuronal excitability, b) decrease glutamate accumulation in the synapse, c) limit calcium mobilization in the postsynaptic neuron and protect against calcium-dependent degenerative effects, and d) enhance neuronal energetic [99].

Control of neuronal excitability

Potassium channels are in charge of controlling the peak of action potentials, i. e. of the control of neuronal excitability. More than one hundred of genes encoding for proteins forming potassium channels have been described [100]. Within this heterogeneity two of those channels are proposed as defense against neuronal over-stimulation. Ca^{2+}-activated potassium channels of small conductance (SK channels) are present in a wide range of excitable and non-excitable cells. On activation by low concentrations of Ca^{2+}, their opening results in hyperpolarization of the membrane potential and changes in cellular excitability [101]. SK channels play a key role in the spike-frequency adaptation and mediate the after-hyperpolarization that causes the refractory period [102]. As potentiation of the after-hyperpolarization by excessive cytosolic calcium would dampen the excitability, SK channels are ideally suited to transduce the calcium mobilization central to excitotoxic injury into a protective, hyperpolarizing signal.

Other candidate to control cellular excitability during excitotoxicity is the ATP-dependent potassium (K_{ATP}) channel, which opening in the hypothalamus is triggered by ATP depletion [103] in the hypothalamus. As a result, K_{ATP} channels can translate the energy depletion induced by any over-stimulation into a protective hyperpolarisation response. Thus, two reliable consequences of excitotoxic insults, the mobilization of cytosolic calcium and the depletion of ATP, would serve to activate potassium channels and decrease neuronal excitability in the face of a receptor over-stimulation.

Decrease of synaptic accumulation of glutamate.

Given the toxic effects of glutamate, adaptations that act to decrease its synaptic accumulation can potentially be protective. A number of the protective mechanisms

against a receptor over-stimulation are conducted to inhibit glutamate release during insults, and some of them involve retrograde signaling of inhibitory neurotransmitters and neuromodulators. Thus, GABA, taurine and adenosine present a retaliatory activity that has shown neuroprotective properties during glutamate-mediated neuronal insults [99]. For example, GABAergic retrograde signaling in hippocampus is multisynaptic, i.e. collaterals from glutamatergic pyramidal terminate on GABAergic interneurons which, in turn, inhibit glutamatergic neurons [104]. Astroglial taurine release during insults derived from potassium and water uptake decreases presynaptic neuronal excitability by increasing chloride influx [105]. Adenosine neuroprotective activity is accomplished through binding to A_1 adenosine receptors linked by G proteins to both calcium and potassium channels [106]. Extracellular adenosine concentration increases after any acute brain injury to exert its protective actions. In this situation, extracellular adenosine increase finally results in an enhancement of uric acid level. Uric acid, a potent antioxidant, preserves mitochondrial activity and acts as a neuroprotective agent against the rise in glutamate induced intracellular calcium concentration [75;79]

Two astroglial sodium-dependent transporters remove synaptic glutamate and transform it into glutamine by means of glutamine synthetase and ATP hydrolysis [107;108]. Glutamine is released and returns to presynaptic neurons, where glutaminase, present prominently in glutamatergic neurons, converts it back into glutamate [109]. The fine adaptation of the glutamate-glutamine cycle to neuronal activity and suffering is important to avoid excessive synaptic glutamate and neuronal death [110-112].

Enhancement of taurine released from glial cells decreases presynaptic excitability by binding to the $GABA_A$ [113] and glycine [114] receptors. By means of these interactions, taurine can help maintain the hippocampal inhibitory tone. In this line, adenosine modulation of glutamate activity also extends to the other systems. An

adenosine modulation of GABA activity has been proposed [81]. This hypothesis is supported by data showing that following ischemia, adenosine receptor agonists inhibit the cortical release of GABA. A reduction in glutamate turnover after adenosine A1 receptor blockade indicates that adenosine participates in the control of the glutamate-glutamine cycle through the modulation of glutamate transport by astrocytes [81]. These studies unveil an interdependency of all these processes, which coordinated adaptation and even the crosslink between their specific pathways are necessary to ensure control of neuronal excitability and receptor over-stimulation.

Limitation of calcium mobilization and protection against calcium-dependent degenerative effects

When cellular calcium homeostasis is overloaded, i. e. the sequestration systems (Ca^{2+}-binding proteins) are saturated and the extrusion ones are activated, Ca^{2+}/Na^{+} antiporters and mitochondrial Ca^{2+} uniporter reduce intracytosolic calcium. When the first wave is stopped, Ca^{2+} binding proteins release Ca^{2+}, which is extruded by the high efficiency cytoplasmatic plasma membrane calcium ATPases. A similar process takes place in mitochondria, the nuclear envelope-endoplasmic-reticulum network, and secretory vesicles (Fig. 4). All these systems, which maintain Ca^{2+} movements under homeostatic control, have a critical dependence on energy. Interplay between all of them constitutes a coordinated way to decrease the extent of calcium mobilization in response to glutamate. Calcium itself can mediate a negative feedback. Calcium-dependent activation of calcineurin and calmodulin can inhibit voltage-gated and NMDA-receptor-gated calcium currents, respectively [99].

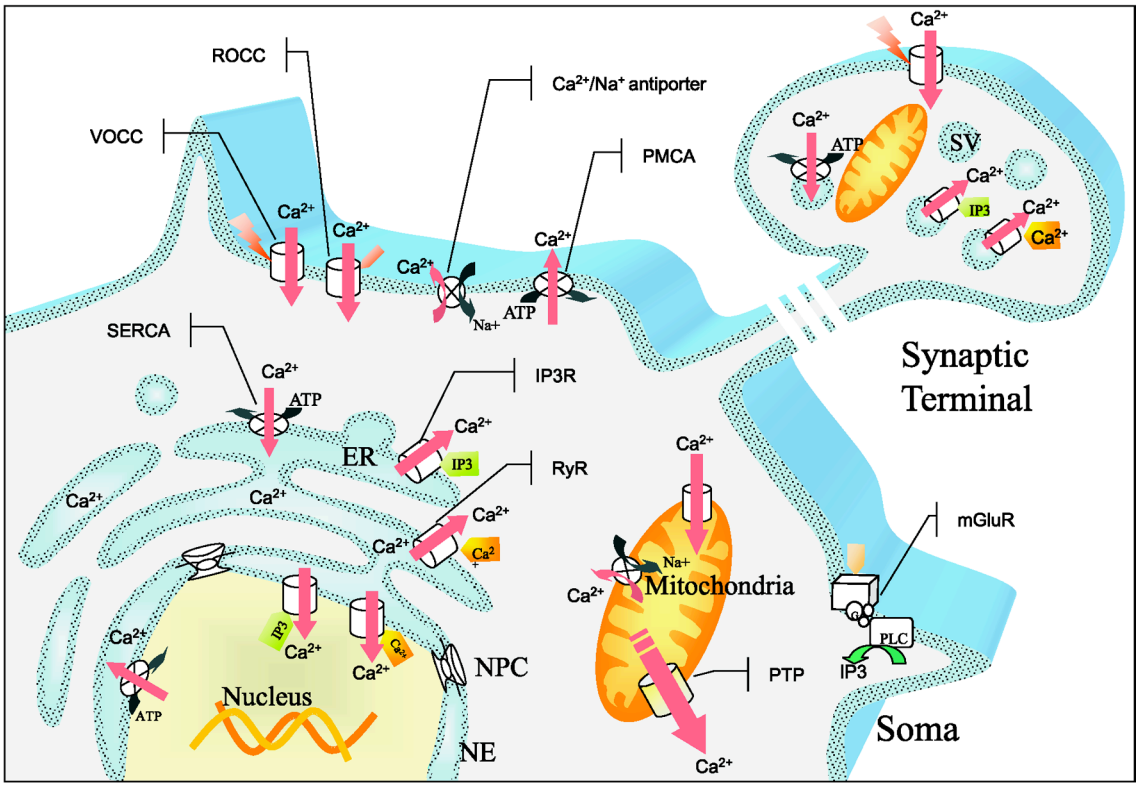

Fig. 4: Schematic diagram of neuronal Ca^{2+} movements. Processes responsible Ca^{2+} extrusion are energy dependent. Processes for increases for cytosolic and nuclear Ca^{2+} are energy independent. (See text for details).

Mitochondrial intake of Ca^{2+} decreases its electrochemical gradient; the opening of the permeability transition pore also dissipates a considerable percentile of membrane potential allowing free circulation of many ions through pores, and all extrusion systems. To restore the loss of electrochemical gradient and global ATP consumption, a fine controlled temporal stimulation of the mitochondrial respiratory chain is required. Any alteration of the energy metabolism affects Ca^{2+} homeostasis and vice-versa [115;116].

Calcium precipitation has been proposed as a putative free-energy defense that limit for calcium movements. Although the significance of cellular calcification is unknown, a number of points suggest that it is part of the compensatory mechanisms for excitotoxic

neurodegeneration. For example, the observation that mitochondria close to Ca^{2+} concretions appear normal at the electron microscopy level supports this hypothesis [71], despite the fact that mitochondrial dysfunction constitutes a primary event in NMDA-induced degeneration in cultured hippocampal neurons [117]. This hypothesis is also consistent with the finding that neurons undergoing prolonged stimulation of NMDA receptors can survive in the presence of $[Ca^{2+}]_i$ chelators. Very high levels of cytoplasmic Ca^{2+} are not necessarily neurotoxic, and an effective uptake of this element into mitochondria is required to trigger NMDA-receptor-stimulated neuronal death [118]. Other results support this hypothesis. In rat globus pallidus, the AMPA-dose-response study has shown a dose-dependent increase in calcification which was not accompanied by an increase in astrogliosis[63]. In hippocampus, AMPA induced a calcified area larger than the injured area. In this same structure, the selective adenosine-A2a-receptor antagonist 8-(3-chlorostyryl)-caffeine increased the NMDA-induced neuronal loss while calcification was decreased [54]. Thus, all these data indicate that Ca^{2+} precipitation does not necessarily reflect neuronal death and that, as proposed for retinal excitotoxic damage [119], besides Ca^{2+} other factors such as Na^+ and Cl^- influx, K^+ efflux and swelling induce excitotoxic neuronal damage.

Excessive $[Ca^{2+}]_i$ ultimately leads to the generation of ROS, cytoskeletal degradation and the misfolding of proteins. A number of adaptations delimit some of these adverse consequences. [99]. All these species can be eliminated by two antioxidant mechanisms: a) molecules such as vitamin C, E, and A, selenium and glutathion; and b) several enzymes like superoxide dismutase, quinone reductase and, the most abundant, astroglial glutathion peroxidase. All of these systems are activated after an acute injury and many of them interfere with glutamate neurotramsmission [99].

Research on calcium precipitation and neuronal suffering is limited, besides the interest of future developments based on the easiness of its detection. For example, in vivo calcification detection would help identify amyloid plaque formation in Alzheimer's disease or stroke, characterize the localization and extension of brain damage after hypoxia-ischemia, and facilitate the follow-up of the lesion [120].

Enhancement of neuronal energetics

Synaptic increase of glutamate level, when not coupled to a heightened energy production, renders neurons susceptible to death. Astrocyte uptake and recycling of synaptic glutamate, as glutamine, is a major pathway dependent on energy metabolism. This dependency, not fully understood remains controversial. Under control conditions, the stoichiometric coupling of glutamatergic activity and glucose metabolism accounts for 80% of total cerebral glucose [91]. Part of this energy is needed for glutamate recycling in a coordinated process involving astrocytes and neurons, with 15% of brain oxidative metabolism contributed by astroglia [121]. Reduced energy availability leading to altered glutamate activity may thus be involved in apoptotic or necrotic neuronal death [87;122]. As revealed by nuclear magnetic resonance studies, glutamate uptake by astrocytes and its return to neurons as glutamine is a major metabolic pathway that reflects most of the cerebral glutamatergic activity [86;91;123]. Finally, the maintenance of lactate deshydrogenase activity in an excitotoxic hippocampal lesion with a 55% of neuronal loss [112], may reflect an astrocyte adaptation to heightened lactate availability to neurons.

Some protective responses to over-stimulation target the energetic vulnerability. At this point, any progress to understand the astroglial contribution for neuronal energy metabolism will be crucial to explain some of these adaptative mechanisms. It is a

general agreement that an increase in glucose transport is protective in brain tissue following an insult. Several pieces of evidence have been reported supporting this statement. The described adaptative mechanisms include an increase in perfusion rate and recruitment of capillaries; stimulation of glial uptake of glucose and glycogenolysis, and an enhancement of glucose uptake and release of adenosine and lactate, increasing lactate metabolic pathway [99]. Thus, after an excitotoxic insult glia takes up glucose and converts it to lactate, which is delivered to neurons as an energy substrate [86].

Uncoupling of the retaliatory systems and energy availability

The tuning between retaliatory system actions and energy metabolism constitutes a fine equilibrium in physiological conditions, but it can be broken by neuronal over-stimulation and then participate of the evoked neurodegenerative process. For example, AMPA-microinjection in medial septum, induces a progressive cholinergic and GABAergic loss associated to a long-term decline of the hippocampal functions [124;125], and decreased glutamatergic activity. Other effects of this lesion imply modifications of adenosine and taurine transmissions, glutamate recycling and glucose metabolism [81;112]. With time, adenosine replaces GABA functions to avoid further excitotoxic damage when cholinergic and GABAergic processes are compromised.

The long-term septal lesion-induced neuronal loss in hippocampus is apoptotic with enhancement of neuronal glycolisis (Fig. 5). Together with a cleavage of caspase 3, a glutamate-glutamine cycle displacement towards glutamine production reduces glutamate synthesis [112]. In addition, synaptic glutamine is decreased, probably expelled to vessels, where it exerts a vasodilatory effect through nitric oxide synthesis inhibition [126]. In this situation the reduction in glutamate signaling and increased neuronal energy metabolism reflect a neurodegenerative process with a deficient

adaptation of the retaliatory systems and a chronic energy requirement to execute the apoptotic program.

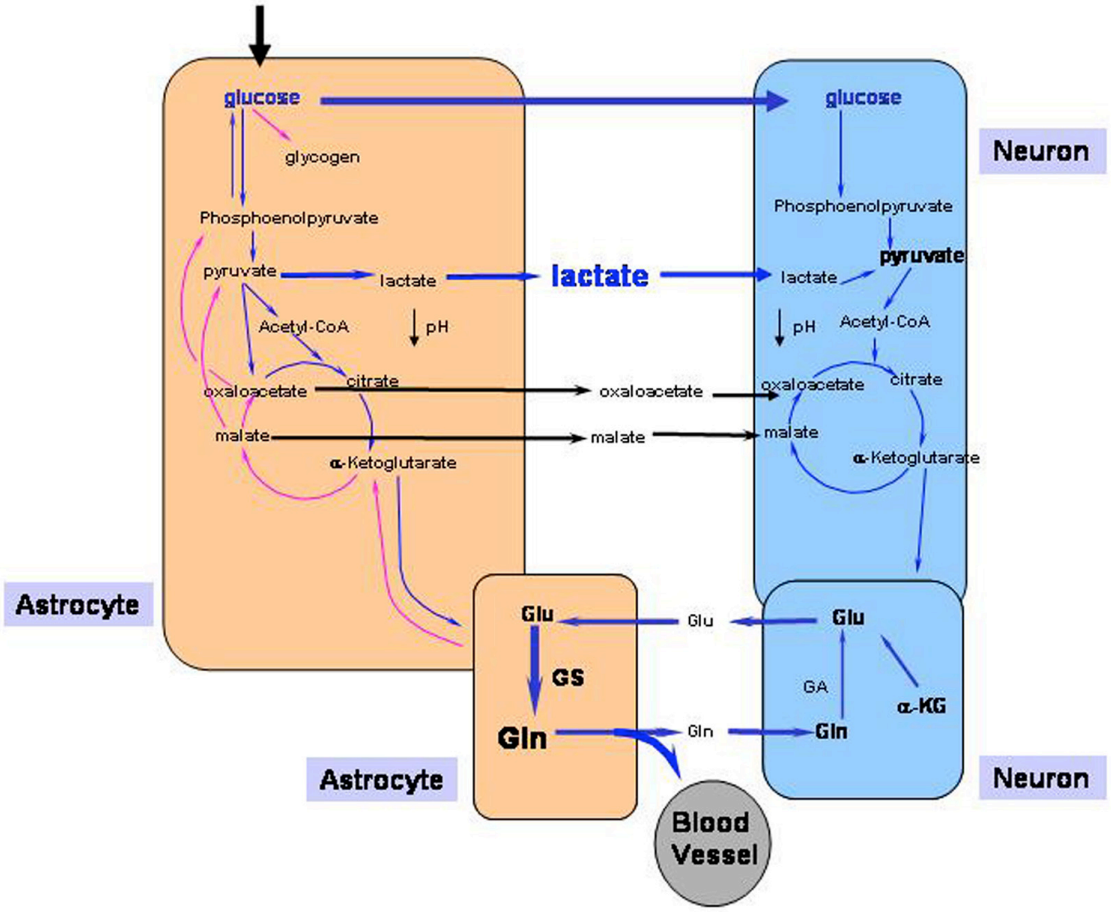

Fig. 5: Schematic drawing of glutamatergic synapse adaptation of the astrocyte-neuron interactions to neurodegeneration. The increased demand of neuronal energy implies a massive lactate formation in astrocytes, a reduced astrocyte activity centered in the uptake of glutamate and glutamine synthesis. Adaptation of the Glu/Gln cycle and neuronal energy metabolism is a key factor in the subsequent demise of the neuron. In physiological situations, increased GA (Glutaminase) activity directly correlates with increased GS (Glutamine synthase) activity, and the cycle recycles glutamate. In neurodegeneration, the GA/GS activities relationship is transformed into an inverse correlation that becomes negative toward a reduced glutamate formation and a net glutamine output. The heightened glutamine production is considered a neuroprotective

adaptation that allows perivascular astrocyte either to remove ammonia or to reduce glutamate released by injured neurons. Increased neuronal glycolysis taking place ultaneously helps sustain the surviving neurons.

ENDURING EFFECTS OF SYNAPTIC OVERSTIMULTION

If the compensatory mechanisms are not effective enough, the initial neuronal acute injury due to $[Ca^{2+}]_i$ increase results, with time, in a chronic lesion. Disturbance of calcium homeostasis is part of all neurodegenerative disorders and *in vivo* and *in vitro* studies have shown an association between Ca^{2+} influx into neurons and neurodegeneration. Dysregulation of Ca^{2+} homeostasis alters the rapid and coherent activation of neurons and therefore is ultimately responsible for many aspects of brain dysfunction and central nervous system diseases. For example, an increased rate of Ca^{2+}-mediated apoptosis may cause neuronal death in the penumbra of cerebral ischemia, or may underlie the etiology of chronic neurodegenerative disorders such as Parkinson and Alzheimer's disease. Calcium precipitation that coincides with microglial activation, amyloid deposits and other ions accumulation in Alzheimer's disease may thus be a key element of the neurodegenerative process.

When an acute brain damage activates a neurodegenerative process its further progression will be related to the intensity of the initial injury. An on-going process with progressive neuronal loss may also be triggered by reiterative sustained neuronal over-stimulation. Acute neurological injury and chronic brain damage has been related in boxing participants, with a correlation in the prevalence of subdural hematoma and dementia pugilistica [127;128]. The same occurs with the disruption of the blood brain barrier induced by epileptic focus that triggers delayed neurodegeneration and functional brain impairment [129]. Cerebrovascular diseases and ischemia-reperfusion

processes might also be central in Alzheimer's disease pathogenesis [130-132]}. Long-term effects of drug dependence underlie an excitotoxicity process linked to a polysynaptic pathway that dynamically regulates synaptic glutamate, and subsequently its dysregulation with modifications of metabotropic, AMPA or NMDA receptor activity. The increased interest in glutamate-based strategies has evidenced promising results [133]. Clinical results with NMDA receptor antagonists such as memantine indicate a decrease of morphine intake in addict; promising results have also been obtained to treat withdrawal syndromes from opioids, alcohol and other sedatives [134]. In addition, blockade of mGluR5 with methyl-6-phenylethymiyl-pyridine may help control the behavioural effects of cocaine, nicotine and alcohol [135-137] and argue for the presence of a chronic neurodegenerative process. However, as said elsewhere, the differences observed in the glutamatergic synapse components in response to acute or chronic exposure to drug abuse and withdrawal are open questions that need to be investigated.

CONCLUSIONS

Variations in CNS acute damage after a similar over-stimulation underlie differences in neuronal populations, abundance and distribution of glutamate receptor subtypes and glial adaptation. This variability determines the induction of a chronic process that develops with distinct neurodegenerative parameters. Thus, at the tissue level, the response to the initial injury can be initially limited by adaptive mechanisms, or produce a variety of lesions related to the neuronal type involved, synaptic density, glial interactions, and vicinity of vascularization. For each neuron and astrocyte type the crew of AMPA/kainate, NMDA and metabotropic glutamate receptors, the Ca^{2+} binding protein content, protein phosphorylation levels, and all elements that participate in

energetic needs and glucose availability will be the factors involved in the appearance of the lesion. Thus, long-term effects of drug dependence are associated with an excitotoxicity process linked to a polysynaptic pathway that dynamically regulates synaptic glutamate. As described in this paper, retaliatory mechanisms include energy capability of the neurons, inhibitory systems and cytoplasmic calcium precipitation as part of the neuron-glia interactions. Their relationship and interdependence help explain the progressive decline of brain functions and bring new targets for therapeutic intervention. However, a better understanding of the complex interactions between cross-link circuits in acute and chronic models of drugs of abuse remains necessary.

ACKNOWLEDGEMENTS

The research projects CIBERNED of the Spanish Ministerio de Sanidad y Consumo, SAF2005-04314 of the Spanish Ministerio de Educación y Ciencia and DURSI 2005SGR00609 of the Generalitat de Catalunya supported this study.

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FUTURE RESEARCH QUESTIONS

The authors consider the following points as the more important questions to answer in future research approaches to determine the cellular and molecular basis of drug abuse and neurodegeneration.

1.- Development of new in vivo models of drug abuse to differentiate between acute and chronic exposure; acute and repeated withdrawal; between low and high dose drug exposure; gender influence

2.- Use of animal models of neurodegeneration to identify and validate new therapeutic targets from glutamatergic neurotransmission and retaliatory systems in acute and chronic drug abuse; acute and repeated withdrawal

3.- Brain calcification and acute and chronic drug abuse

4.- CNS development, Aging and drug addiction

5.- Female versus male in development of drug addiction and withdrawal

KEY OBJECTIVES

This review deals the following learning objectives:

1.- To understand the cellular and molecular types of adaptation to long-lived aspects of addiction and withdrawal

2.- To establish the relationship between acute drug abuse, formation of calcium precipitates and neuronal death.

3.- To establish the relationship between drug enduring effects, activation of glutamate receptors, glia response and neurodegeneration.

4.- To get an integrated view of how a drug interferes the energy capability of neurons, the neuron-glia interactions and the inhibitory systems aimed at the control of acute and chronic over-stimulation.