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Calcium homeostasis in the central nervous system: adaptation to neurodegeneration

Manuel J. Rodríguez, Rosa Adroer, Lluïsa de Yebra, David Ramonet and Nicole Mahy*
Unitat de Bioquímica, Facultat de Medicina, IDIBAPS**, Universitat de Barcelona

Abstract

Here we review the results of our recent studies on neurodegeneration together with data on cerebral calcium precipitation in animal models and humans. A model that integrates the diversity of mechanisms involved in neurodegeneration is presented and discussed based on the functional relevance of calcium precipitation.

Resum

En aquest article, després d'una revisió dels nostres coneixements bàsics sobre moviments del calci neuronal, s'ha presentat el treball fet pel nostre grup durant els últims anys sobre neurodegeneració, juntament amb les dades obtingudes en models animals i humans en l'estudi de la precipitació cerebral del calci. Per tal d'explicar la precipitació del calci s'ha presentat i discutit un model que integra els diversos mecanismes implicats en neurodegeneració des del punt de vista de la rellevància funcional.

Keywords: Calcium, brain, neurodegenerative disease, glutamate, excitotoxicity

Part 1. Calcium homeostasis in the CNS: a general overview

1.1. Organization

In neurons, calcium (Ca^{2+}) plays a central role both as a charge carrier, capable of regenerative electroresponse, and as second messenger in cytosolic and nuclear transactions. As second messenger, Ca^{2+} transmits signals through its complexation by specific proteins, inducing conformational changes that result in the regulation of phenomena involved in cell plasticity and survival. Neuronal activity therefore depends on a fine temporal and spatial tuning of Ca^{2+} movements on an electrochemical gradient, the buffering capacity of fixed and mobile Ca^{2+} binding proteins and the activity of membrane-intrinsic Ca^{2+}-transport systems. As the intracellular mobility of Ca^{2+} is the most restricted of all the abundant cellular ions, oscillations result in intracytoplasmic concentrations in microdomains that trigger and control reaction cascades. These cascades regulate neuronal events such as neurosecretion, formation of resting and action potentials, long-term potentiation (LTP), apoptosis and neuronal death [94,95], or the rescue of the latter [25]. Rapid regulation of intraneuronal Ca^{2+} movement is crucial, allowing a punctual and localized increase, followed by a rapid recovery of initial levels [12].

Consequently, 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 CNS 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 Alzheimer's or Parkinson's diseases.

1.1.1. Compartmentalization of neuronal calcium

Initiation of Ca^{2+} neuronal signalling is controlled by two types of excitable media; by the cellular membrane, which dominates, and by specialized intracellular organelles or compartments, which also participate in initiating, shaping and integrating calcium signals. Together with the synaptic cleft, each compartment has its own loading capacity and acts as a calcium source or drain under the control of specific proteic mechanisms which quite often coexist as different isoforms in the same neuron. Neuronal Ca^{2+} movements occur in four compartments: a) the synaptic cleft, b) the cytoplasm and nucleoplasm, c) the endoplasmic reticulum (ER)

* Author for correspondence: N. Mahy, Unitat de Bioquímica, Facultat de Medicina, Universitat de Barcelona. Casanova 143. 08036 Barcelona, Catalonia (Spain). Tel. 34 93 4024525. Fax: 34 93 4035882. Email: mahy@medicina.ub.es

** IDIBAPS: Institut d'Investigacions Biomèdiques August Pi i Sunyer

(mainly the smooth one), the nuclear envelope (NE), and secretory vesicles (SV), and d) the mitochondria. These compartments are delimited by three barriers which have specific gates that accumulate Ca^{2+}, maintain it for a long period of time, and release it in response to a stimulus. The first barrier corresponds to the plasmatic membrane whose gates are specific receptor– and voltage-operated Ca^{2+}channels (ROCCs and VOCCs), a Ca^{2+} ATPase and a Na^{+}/ Ca^{2+} exchanger. The second barrier is composed by the topologically-related ER, NE and SV membranes which have inositol trisphosphate (IP_3) and ryanodine receptors operated channels and a Ca^{2+} ATPase. The mitochondrial membrane is the third barrier and has a Ca^{2+} uniporter, a Na^{+}/ Ca^{2+} exchanger and a permeability transition pore (PTP) (See Figure 1 and Table 1).

The concentric double membrane of the NE delimits nuclear cisterna, with the outer membrane physically and functionally continuous with the ER, and the inner membrane interacting with skeletal nuclear components. Both membranes are traversed by nuclear pore complexes (NPC) of around 125,000 kDa [11] through which ions and small molecules (<500 Da) diffuse freely [44]. Proteins extending from the NPC into the NE cisterna contain Ca^{2+} binding sites, so the Ca^{2+} of the NE lumen and the nucleoplasm results in conformational changes that control the opening or blockade of the pore, thereby preventing the passage of medium sized molecules [11,44]. Furthermore, as a dynamic supramolecular assembly [44] under the control of nuclear localization signals [11], NPC accommodate their resting 9 nm diameter to the transport of large molecules (>70 kDa), which can reach up to 26 nm in ATP-activated ones [24,44].

In each compartment, large amounts of Ca^{2+} binding proteins (parvalbumin, calretinin, calbindin, etc.) with a Kd<1μM allow lumen storage of Ca^{2+}, leaving less than 1% in its free ionic form. In most of these proteins Ca^{2+} binding results in limited conformational modifications which have no direct effect on any neuronal process [33]. These modifications explain the buffering role of these proteins. Although most Ca^{2+} movements are directed to the cytosol, once the stimulus-evoked Ca^{2+} release has been stopped, the presence of well-coordinated high affinity/capacity buffers and transport mechanisms ensures a rapid reduction of Ca^{2+} content, thereby restoring the resting potential (i.e. from mM to nM in less than 10 s) [8,40,57,58,91].

A general overview of the localization of these multiple elements is shown in Figure 1. Table 1 summarizes the main characteristics of each buffer and transport mechanism.

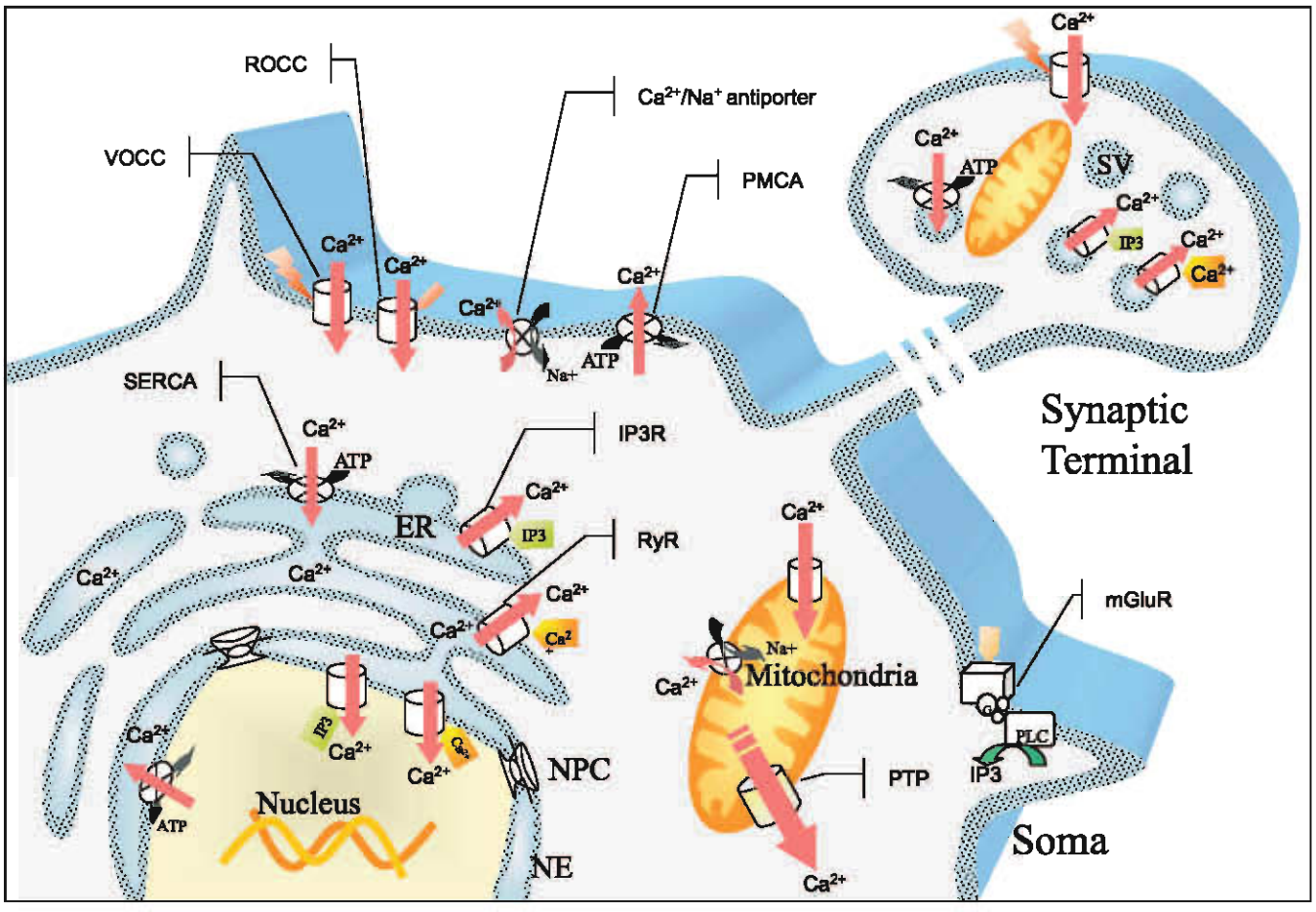
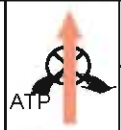


Figure 1: Schematic diagram of neuronal Ca^{2+} movements. Processes responsible for Ca^{2+} extrusion are energy dependent. Processes for increases in cytosolic and nuclear Ca^{2+} are energy independent (See Table 1 for legend).

Table 1.

	Legend	Nomenclature and subtypes	Transport characteristics	Process type	Endogenous & exogenous modulators	CNS tissue distribution	Subcellular localization
Non-Energy depending processes		L-type VOCC	Extra-cellular calcium intake into cytosol. May create microdomains.	Voltage operated calcium channels.	PKC phosphorilation increases their probability of aperture. Inhibition by Gi/o proteins. There are many pharmacological important blockers (e.g. DHPs). Bay K-8644 is an activator	Wide distribution; in CNS is present in neurons and astroglia.	Plasmatic membrane except axons and synaptic terminations
		N-type VOCC	Extra-cellular calcium intake into cytosol.		Irreversible blockage by GVIA w-conotoxin, reversible blockage by MVIIC w-conotoxin.	Wide distribution; mainly in neurons.	Plasmatic membrane including synapse.
		P-type VOCC (also called P/O type)	Extra-cellular calcium intake into cytosol.		Irreversible blockage by IVA w-conotoxin, reversible blockage by MVIIC w-conotoxin. Inhibition by Gi/o proteins.	Purkinje cells of cerebellum and some neural subpopulations.	Plasmatic membrane including synapse.
		NMDA receptors (NMDAR): dimer NMDAR1 + NMDAR2a-d; four NMDAR2 isoforms with multiple trans-splicing variants.	Late extra-cellular calcium intake into cytosol.	Receptor operated calcium channel activated by Glu,Asp,NMDA and ibotenic acid. Also permeable to other ions.	Coagonism by Glycine is necesary for activation. Zn^{2+},H^{+} and poliamines are alosteric factors. Mg^{2+} is a reversible blocker. Multiple agonists and antagonist known.	Neurons (and immature astrocytes) of cortex, hippocampus and nucleus acumbens. Other structures too.	Postsynaptic membrane.
		AMPA/KA receptors; only subtype w/ GluR1 & w/o GluR2 is mainly permeable to calcium.	Early extra-cellular calcium intake into cytosol.	Receptor operated calcium channel activated by Glu, AMPA and kainate. Also permeable to sodium.	Multiple agonists and antagonist known.	Astrocytes and GABAergic neurons expressing parvalbumin.	Plasmatic membrane including synapse.
		IP3 receptors (IP3R); 3 isoforms.	Release stored calcium to cytosol.	Receptor operated calcium channel activated by IP3	Activation by low cytosolic [Ca^{2+}] and by high luminal [Ca^{2+}] conc. Inhibition by high cytosolic [Ca^{2+}]	Wide distribution	ER and vesicular membranes, nuclear envelope
		Ryanodine-cADP ribose receptors (RyR); 3 isoforms	Release stored calcium to cytosol.	Receptor operated calcium channel activated by cytosolic calcium.	Activation by high luminal Ca^{2+}. Cyclic ADP-ribose is a co-agonist. CaMKII phosphorilation and high cytosolic ATP are positive modulators.	Wide distribution	ER and vesicular membranes, nuclear envelope
		Permeability transition pore (PTP); complex structure.	Slow release calcium to cytosol in the reversible low conductivity state.	Transmembrane pore permeable to many ions and small substances.	Ciclosporin A is a blocker. Matrix calcium, oxidative stress and intramitochondrial glutation decrease promote its opening.	Wide distribution	Mitochondrial membrane
Energy- depending processes		Sarco(endo)plasmatic calcium ATPases (SERCA); 3 isoforms	Internalize cytosolic calcium into ER.	High performance calcium pump	Activation by high cytoplasmatic calcium concentrations, inhibition by high ER conc.	SERCA2b is the most common in CNS; SERCA3 in cerebellum.	ER and vesicular membranes, nuclear envelope
		Plasma membrane calcium ATPases (PMCA); 5 genes, 9 complex trans-splicing variants.	Extrude calcium from cytosol to extra-cellular space	High affinity low capacity calcium pump	Alosteric positive modulation by calmodulin (Km 10 -> 0.3 μM) Inhibition by TMB-8	PMCA 1,2,3 are widely expressed in CNS.	Plasmatic membrane
		Ca^{2+}/3Na^{+} antiporter; 3 genes (NCX1-3)	Extrude calcium from cytosol to extra-cellular space	Low affinity high capacity antiporter, it is coupled to Na^{+}/K^{+} pump	Alosteric positive modulation by ATP (Km 10-15 -> 1-3 μM)	Wide distribution, NCX3 only in brain.	Plasmatic membrane
		Ca^{2+}/3Na^{+} antiporter	Extrude calcium from mitochondrial matrix to cytosol.	Slow and low capacity antiporter, is coupled to slow Na^{+}/H^{+} antiporter.	Inhibition by CGP-37157 and respiratory uncouplers as CCCP and FCCP.	Wide distribution	Mitochondrial membrane
		Ca^{2+} mitochondrial uniporter.	Internalize cytosolic calcium into mitochon-drial matrix.	Low affinity electrogenic uniport.	Is only active in non-resting high cytosolic [Ca^{2+}],being positively modulated by calcium itself and poliamines like spermine. Sr^{2+} & Ba^{2+} are competitive inhibitors.	Wide distribution	Mitochondrial membrane

1.1.2. Extracellular calcium

In neuronal cells, the entry of extracellular Ca^{2+} depends on ROCCs and VOCCs. The former include the two ionotropic glutamate receptor subtypes, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) lacking the GluR2 subunit, and N-methyl-D-aspartate (NMDA), each with selective agonists and antagonists [70]. VOCCs are highly selective for Ca^{2+} and non-permeable to monovalent ions, and are classified into five subtypes (T, L, N, P and Q), following their pharmacological and electrophysiological properties [35]. The T subtype is unique in its capacity to open briefly after a weak depolarization of a membrane [71], while the other types need a greater depolarization. A high density of the L subtype in dendrites may result in large localized increases in Ca^{2+}, thereby creating microdomains. ATP associated to P2 purinergic receptors may regulate Ca^{2+} entrance [48] and thus directly link neuronal electrical activity with theenergetic state of the cell. Finally, reversion of the $Ca^{2+}/(Na^{+})$ antiporter takes place after intense depolarization, resulting in the opposite movement, i.e. the entrance of Ca^{2+} [34].

In astrocytes, cytosolic Ca^{2+} movements are controlled by similar compartmentalized intracellular mechanisms. ROCCs and VOCCs are present to a lesser extent, namely through AMPA and P2 receptors. VOCC expression is highly heterogeneous, the T– and L-type genes being the most expressed [98].

1.2. Calcium homeostasis

The synaptic glutamate release and activation of ionotropic (NMDA or AMPA) or metabotropic receptor lead to increases in Ca^{2+} current. This results in membrane depolarization and activation of several cytosolic and nuclear processes. The concentration of synaptic glutamate and the activated receptor subtype determine neuronal Ca^{2+} mobilization, resulting thus in differences in neuronal responses (Figure 2).

Activation of ionotropic receptors allows the direct entrance of extracellular Ca^{2+}, whereas metabotropic receptors linked to phospholipase C (PLC) through the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglicerol (DAG) and IP_3, activate the receptor-induced Ca^{2+} release in the ER, NE and SV. Therefore, the microdomains of high Ca^{2+} content that are close to the ER sites of Ca^{2+} release activate rapid mitochondrial uptake through an electrogenic uniporter. This uptake leads to Ca^{2+} stimulation of pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase, resulting in an increase in neuronal respiratory capacity [92] (Figure 2).

Ca^{2+} from the ER, NE and SV can also be released through the ryanodine receptor, which is sensitive to cyclic adenosine diphosphate ribose (cADPR). This receptor has a certain degree of homology with the IP_3 receptor [56] and is the main responsible for calcium-induced calcium release (CICR) [42,98]. Initial Ca^{2+} influx through NMDA receptors, VOCCs or mGluR-activated IP_3 receptors, activates RyRs and releases Ca^{2+} from cell stores. Therefore, this initial increase in Ca^{2+} content results in a temporally and spatially displaced higher concentration, which in turn activates another RyR. Consequently, a Ca^{2+} wave is rapidly generated and propagated through the soma, nucleus and axon, which send the initial signal away from its origin. As a micromolar concentration of cytoplasmic Ca^{2+} is needed to trigger this signal, CICR initiation occurs only after a burst of action potentials, except in some particular situations. However, not only the amount of Ca^{2+}, but also its increase is crucial for CICR, especially near the release sites. IP_3 receptors prevail in astroglial cells where they may be critical for the induction of Ca^{2+} waves through gap junctions. Furthermore, the presence of functional ryanodine receptors is controversial [98].

The autonomous regulation of nuclear Ca^{2+} has been suggested [13]. Nuclear-specific Ca^{2+} signals must be initiated by Ca^{2+} release channels in the inner membrane of the NE [11]. In the cytosol, the generation of IP_3 is the focal point for intracellular Ca^{2+} signalling. Two interconnected pools of PIP_2 have been identified in the nucleus, one is probably present in the NE and the other is located within the nucleus, either as part of a proteolipid complex, or of the recently identified invaginations of the NE, which invade the inner matrix of the nucleus [54]. With their dynamically changing morphology, these NE tubules or invaginations could provide a source of Ca^{2+} signals deep inside the nucleus [11]. Other components required for the nuclear inositide cycle are present in the nucleus, including phospholipase Cβ1, which hydrolyzes PIP_2 to generate nuclear IP_3 and diacylglycerol [54].

The entrance of nuclear Ca^{2+} can also be mediated by inositol 1,3,4,5-tetrakisphosphate (IP_4) or by ATP. IP_4-mediated Ca^{2+} uptake occurs above a concentration of 1 μM free Ca^{2+} but its energetic cost remains unknown. In the cytosol, the role of IP_4 is also poorly understood. Nuclear IP_4R is a 74 kDa protein that differs from other IP_4R so far reported [54].

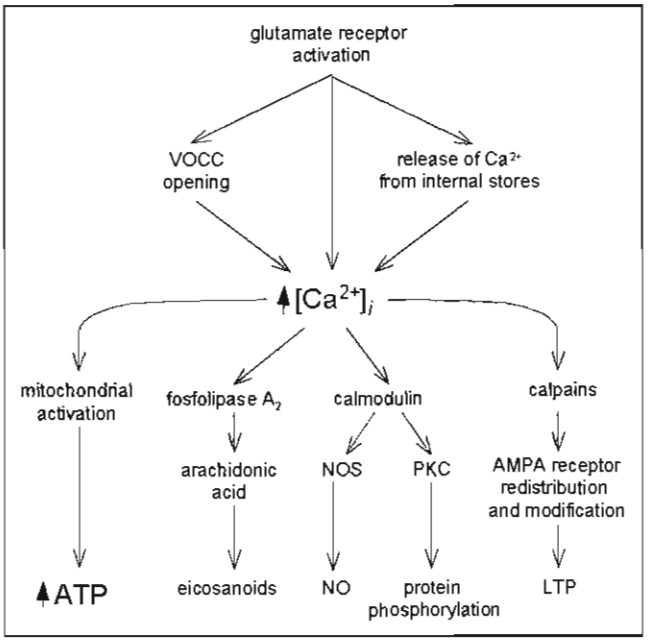


Figure 2: Post-synaptic mechanisms activated at the glutamatergic synapse. The glutamate-mediated increase in intracellular Ca^{2+} can mostly activate five pathways. NOS, nitric oxide syntase; PKC, protein kinase C.

NPC or Ca^{2+}-ATPase on the inner nuclear membrane may mediate the extrusion of Ca^{2+} from the nucleoplasm [54]. Finally, to control cisternal Ca^{2+} stores in the nucleus, a Ca^{2+}-ATPase traverses a single bilayer, which is identical to the Ca^{2+} pump in the ER [44].

At rest, the extracellular medium and the inside of NE-ER network are characterized by > 100 μM Ca^{2+}. In contrast, the cytosol and the nucleoplasm have a lower concentration, around 100 nM. Locally, after stimulation, the Ca^{2+} content close to clusters of IP_3 or cADPR receptors may rise transiently to a few μM. Ca^{2+} signals generated in the nucleoplasm close to the Ca^{2+} release channels in the inner nuclear membrane may be quickly dissipated because of diffusion of this ion through the permeable NPCs and subsequent dilution into the cytoplasm, as well as its entrance into the ER. Any increase in cytosolic Ca^{2+} then activates a pump in the plasma membrane [73] which extrude Ca^{2+} into the extracellular medium. Similarly, depletion of Ca^{2+} in the NE-ER store opens store-operated Ca^{2+} channels in the plasma membrane, allowing its entry. Because of the rapid local uptake of Ca^{2+} into the ER, this process does not necessarily alter cytosolic concentrations of this ion [73].

When the system is overloaded (as in sustained CICR or tetanizing activation), Ca^{2+}-binding proteins are saturated and extrusion is activated. Ca^{2+}/Na^{+} antiporters, and mitochondrial Ca^{2+} uniporter reduce intracytosolic Ca^{2+}. 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 (PMCAs). A similar process takes place in mitochondria, the NE-ER network, and SV.

The cytosolic membrane has two complementary systems that extrude Ca^{2+} to the extracellular space, PMCAs and Ca^{2+}/Na^{+} antiporters. PMCAs are responsible for maintaining the resting ionic gradient; they have a high affinity and a low capacity for Ca^{2+}, and are alosterically activated by calmodulin (their K_M decreases from 10 to 0.3 μM). In contrast, Ca^{2+}/Na^{+} antiporters have a low affinity but a high capacity and are alosterically activated by ATP (their K_M decreases from 12 μM to 2 μM). This electrogenic antiporter (3 Na^{+} for 1 Ca^{2+}), which is also present in mitochondria, is driven by a gradient sustained by the Na^{+}/K^{+} ATPase. In depolarization this system can be inverted [34].

Ca^{2+} internalization at the ER-NE is due to a family of high performance Ca^{2+}ATPases or SERCA –sarco (endo) plasmatic reticulum calcium ATPases– which are present as three proteins: SERCA2a, SERCA2b and SERCA3. All three are activated at high cytoplasmic Ca^{2+} concentrations and are inhibited at high free Ca^{2+} content in the ER. To avoid this inhibition, Ca^{2+} binding to specific ER Ca^{2+} binding proteins, such as calsequestrin, can maintain a low reticular concentration.

Thus, Ca^{2+} movements have a critical dependence on energy. Mitochondrial intake of Ca^{2+} decreases its electrochemical gradient; the opening of PTP also dissipates a considerable percentile of membrane potential allowing free circulation of many ions through pores, and all extrusion systems need ATP directly or indirectly. 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 [5,8,59].

1.3. Calcium and gene expression

The primary genomic response to a variety of external signals is the rapid induction of a set of genes named immediate-early response genes (IEGs). Studies have implicated these genes in processes such as oncogenic transformation, cellular growth and differentiation, and synaptic plasticity [76]. Only a few IEGs are known to be induced by Ca^{2+} and evidence suggests that it inhibits the expression of some IEGs [76].

In PC12 cells, several IEGs are Ca^{2+}– inducible. The most notable include the *c-fos* and *c-jun* proto-oncogenes and *junB*. Because of its very rapid and pronounced induction by a variety of agents in different cell types, the *c-fos* gene has served as a paradigm of early response gene regulation [76]. Increasing intracellular Ca^{2+} produces the transcriptional induction of the *c-fos* gene. This gene contains two Ca^{2+} response elements in its promoter, the cAMP response element (CRE) and the serum response element (SRE). Cytoplasmic Ca^{2+} targets the SRE while nuclear Ca^{2+} controls gene expression through the CRE [30].

Ca^{2+}-activated transcription mediated by CRE and CRE-binding protein (CREB) requires an increase in nuclear Ca^{2+} content. This observation indicates that the machinery responsible for this response is in the nucleus. The ability of CREB to activate transcription requires its phosphorylation on Ser 133, a reaction catalyzed by various protein kinases. Calmodulin kinase IV, localized in the nucleus, is involved in nuclear Ca^{2+}-activated transcription and can activate CREB-mediated transcription [30]. The first step is Ca^{2+}-induced phosphorylation of CREB on Ser 133, which leads to recruitment of the transcriptional co-activator CREB binding protein (CBP) to the promoter. The second regulatory event, which is crucial for transcriptional response, stimulates CBP activity (or a CBP associated factor). Both events can be mediated by CaM kinase IV. An additional degree of complexity of Ca^{2+} signalling on IEG expression has been adduced recently in hippocampal neurons where several types of Ca^{2+} channels are linked to distinct signalling pathways. Activation of CaM kinase in *c-fos* induction appears to be critical only for the Ca^{2+} that enters via the L-type channel. In contrast, NMDA receptor-operated Ca^{2+} channels use, in addition, a distinct pathway to induce *c-fos*, which may involve tyrosine phosphorylation signalling and the MAP kinase cascade [76]. Another family of the so-called basic-helix-loop-helix (bHLH) transcription factors has been implicated in gene regulation by Ca^{2+} [76]. Calmodulin, calcineurin, and certain S-100 proteins play a key role in this process [54].

Recently, it has been described for the first time that a nuclear Ca^{2+} sensor, DREAM (Down-stream Regulator Element Antagonist Modulator), directly represses transcription by

binding specifically to DNA. DREAM's affinity for DNA is reduced upon binding to Ca^{2+}. This represents a direct mechanism for Ca^{2+}-induced gene expression that is not dependent on changes in the activity of other transcriptional effectors through phosphorylation or protein-protein interactions. Target genes for DREAM repression include prodynorphin and *c-fos*, indicating that DREAM may have a role in the regulation of a broad range of genes [15].

1.4. Protease activation, LTP and neuronal plasticity

LTP is characterized by a persistent increase in synaptic efficacy resulting from repetitive, high frequency stimulation of afferent fibers. This phenomenon was first demonstrated in hippocampus and has since been shown to occur in most limbic structures and cortex. An increasing number of experimental results support the hypothesis that the substrates underlying LTP are related to learning and memory processes [66]. Induction of LTP in the CA1 hippocampal region involves: (1) activation of post-synaptic NMDA receptors and (2) an increase in intracellular Ca^{2+} concentration (Figure 2). This increase presumably results in the activation of Ca^{2+}-dependent enzymes of post-synaptic cells that ultimately produce stable potentiation [21]. Calpains are required for LTP induction, but they are not required to be constitutively active for LTP maintenance [22].

Whereas LTP induction requires activation of NMDA receptors, LTP expression and maintenance are presumed to be due, in part, to modifications of AMPA receptors. It has been proposed that Ca^{2+} influx through the NMDA receptor channel activates calpain, which cleaves cytoskeletal proteins (including spectrin), thereby resulting in the redistribution and modification of AMPA receptors in the post-synaptic membrane. In addition, stimulation of the NMDA receptor produces an accumulation of a specific spectrin breakdown product (SBDP) which is generated by calpain cleavage of spectrin. Other processes, such as phosphorylation reactions mediated by a variety of protein kinases, have also been proposed to participate in synaptic plasticity [28]. It has been suggested that cleavage of the C-terminal domain of GluR1 by calpain results in increased AMPA receptor function and this mechanism also participates in LTP expression [61] (Figure 3).

Many of the signals involved in developmental and synaptic plasticity in the nervous system are also involved in neuronal death in both physiological and pathological settings. Two prominent examples are the signal transduction pathways activated by neurotrophins and the excitatory neurotransmitter glutamate. Glutamate-mediated Ca^{2+} increase may activate the calpain and caspase families, which play key roles in modulating synaptic plasticity and in apoptotic neuronal death [16].

1.4.1. Calpains

Calpains are Ca^{2+}-activated cysteinyl/thiol proteases with two ubiquitous isozymes expressed as proenzyme heterodimers, calpain I with a high affinity for Ca^{2+}, and calpain II with a low affinity. Within the brain, the former is mainly neuronal and is often active constitutively, with higher levels in dendrites and soma, whereas calpain II predominates in axons and glia and is generally activated in response to cellular signals [16].

The activation of calpains is stimulated by an increase in intracellular free Ca^{2+}, and is inhibited by the protein calpastatin and by a decrease in intracellular Ca^{2+}. Calpain I may be activated in the cytosol or when bound to the plasma membrane, whereas the activation of calpain II occurs mainly at this membrane. Upon binding to Ca^{2+}, calpains undergo a conformational change and translocate to phospholipid

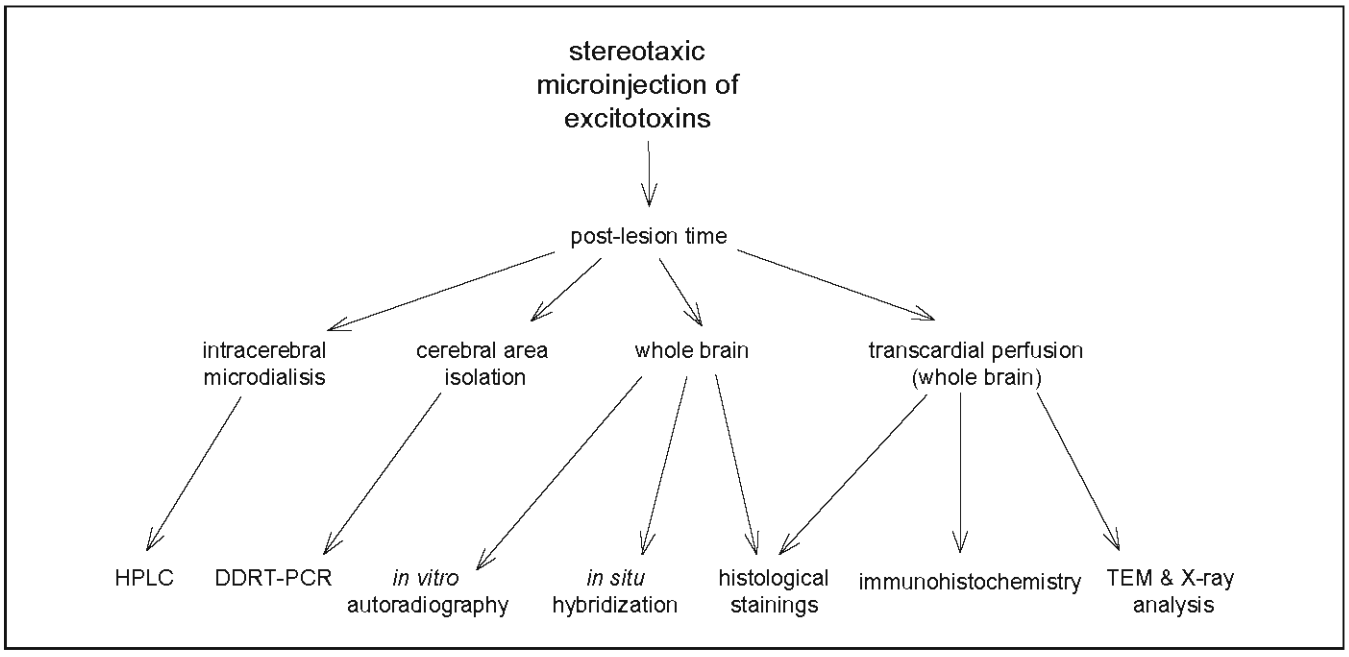


Figure 3: Methodological approach used to study our models of neurodegeneration. Stereotaxic acute microinjections of several compounds were performed in several areas of rat brain, and the post-lesion time varied between 13 days and one year. HPLC, high performance liquid chromatography; DDRT-PCR, differential display reverse transcriptase-polymerase chain reaction.

membranes where limited autolysis of the N-terminus of both subunits occurs. Caspase also degrades calpastatin and consequently leads to increased calpain proteolytic activity.

This model of calpain activation proposes that attachment to plasma membrane sites increases Ca^{2+} sensitivity, facilitating autocatalytic conversion of calpain at physiological concentrations of this ion (100-1000 nM). The autolytically activated protease requires a lower concentration of Ca^{2+} for its activation [16].

1.4.2. Caspases

Lack of trophic support and activation of glutamate receptors can induce proteolytic caspase activation in neurons. Pro-caspases are constitutive neuronal enzymes whose activity is regulated through many mechanisms. The catalytically active caspase is a tetrameric complex formed by multimerization of pro-caspase molecules during processing and activation. The caspase family can be grouped into two classes: initiators and effectors. A rapid and effective processing of substrate is ensured by the redundancies which are common among caspases, many having the ability to cleave the same substrates. Caspase activation may be determined by their subcellular localization (membrane-bound, endoplasmic reticulum, mitochondrial intermembrane space and extracellular surface of the plasma membrane) [16]. Physical interaction with cellular proteins (like the inhibitor of apoptosis proteins (IAPs) and Bcl-2, the most-studied negative regulator of neuronal apoptosis) is another way to regulate caspase activation and activity. Overexpression of Bcl-2 protects against a range of apoptosis-inducing stimuli, including trophic factor withdrawal, glutamate, oxidative insults, glucocorticoids, and DNA-damaging agents [16]. Post-translational modification and phosphorylation also regulate caspase activation. In addition, nitric oxide (NO) and reactive oxygen species can modulate this activity [84] and inhibit apoptosis. The potent anti-apoptotic activity of NO is attributed to its ability to reversibly inhibit the enzymatic activity of caspases by direct S-nitrosilation of the catalytic cysteine residue that is essential for enzyme activity [80]. As caspase activation does not necessarily lead to apoptosis, it is important to bear in mind the physiological roles for caspases in synaptic plasticity. Thus, because apoptosis is an irreversible phenomenon, caspase disactivation following non-apoptotic stimuli is ensured by several endogenous caspase inhibitors, some of which are also encoded by oncogenes and viruses [16].

Part 2. Neurodegeneration as a result of disturbances in calcium homeostasis

Intracellular Ca^{2+} overload may injure the CNS. Several studies *in vivo* and *in vitro* show an association between Ca^{2+} influx into neurons and neurodegeneration. For example, an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) participates directly in the neuronal damage observed during cerebral ischemia and epileptic seizures [64,96]. However, the mechanisms by which Ca^{2+} exerts its neurotoxic actions remain unclear. It has been proposed that $[Ca^{2+}]_i$ increase may over-stimulate cellular processes that normally operate at low levels, or may trigger certain cascades which are not usually operative [96]. Some of the mechanisms directly activated by Ca^{2+} or Ca^{2+}-calmodulin include energy depletion due to a loss of mitochondrial membrane potential, production of reactive oxygen species (ROS), formation of membrane gap by over-activation of phospholipase A_2, calpain-induced cytoskeleton breakdown, and endonuclease-mediated DNA degradation [59,69].

2.1. Calcium-mediated excitotoxicity

A link between $[Ca^{2+}]_i$ increase, over-activation of excitatory amino acid (EAA) receptors, and neuronal death has been established from data obtained in models of neuronal cultures, hypoxia-ischemia and after specific stereotaxic cerebral lesions [18,26,96]. In these experimental models, the induced neurodegeneration can be blocked using intracellular Ca^{2+} chelators [97] or VOCC antagonists [49], removing extracellular Ca^{2+} [27], or emptying cellular Ca^{2+} stores [45]. Thus, neurodegeneration can start after an acute injury, such as a hypoxic-ischemic episode or a traumatic lesion, whose immediate effects depend on a diversity of Ca^{2+} activated lesioning and compensatory mechanisms. If sufficient, the initial insult results in a chronic on-going process, with a progressive loss of neurons. In the early 1970s, Olney defined excitotoxicity as the neuropathological process triggered after over-stimulation of EAA receptors [67]. At present, excitotoxicity includes the concept of glutamate-mediated endogenous neurotoxicity; i.e. the putative excitotoxicity when glutamate increases in the extracellular space [65]. This concept is of interest because it presents the possibility of new strategies in pharmacological neuroprotection.

Thus, it is possible to develop animal models of neurodegeneration which are based on controlled glutamate receptor over-stimulation in a selected area of the CNS while keeping the animal alive for some time. We used stereotaxic microinjections of EAA receptor analogs, such as NMDA, AMPA, ibotenic and quisqualic acids, to induce an excitotoxic lesion in brain areas of rat that are related to neurological diseases in humans (Figure 3).

2.2. Chronic excitotoxic lesion

Because of the complexity and diversity of the processes taking place at glutamatergic synapse, any anomaly at the pre-synaptic, post-synaptic, or astroglial level may trigger a chronic excitotoxic process. For example, a loss of selectivity of ionotropic receptors [65], or deficiencies in glial re-uptake of glutamate [46] are observed in lateral amiotrophic sclerosis. These dysfunctions contribute to explain phenomena such as the aging-associated hypoactivity of NMDA receptors observed in Alzheimer's disease [68] and the AMPA-receptor increment detected in the hippocampus of aged-impaired rats [43]. We are currently using a diversity of *in vitro* and *in vivo* techniques to further explain these molec-

ular and cellular mechanisms involved in neurodegeneration (Figure 3).

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. Cerebral lesions can be characterized by the area of lesion, neuronal death, and the astro– and microglial reactions. The excitotoxic injury in neurons appears after the massive entrance of Ca^{2+} and Na^+ through ionotropic glutamate receptors [18], this entrance is supplemented by the Ca^{2+} release from the ER after activation of mGluRs. As a result, an excessive $[Ca^{2+}]_i$ increment occurs [18], which activates the mechanisms triggering neuronal death (Figure 4). Ca^{2+} extrusion and buffering are activated when the $[Ca^{2+}]_i$ increases [96,99], 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 ROS, thereby decreasing cellular respiratory capacity [89]. As a result, aerobic glycolysis accelerates during the period soon after acute excitotoxicity; however, because of the mitochondrial injury,

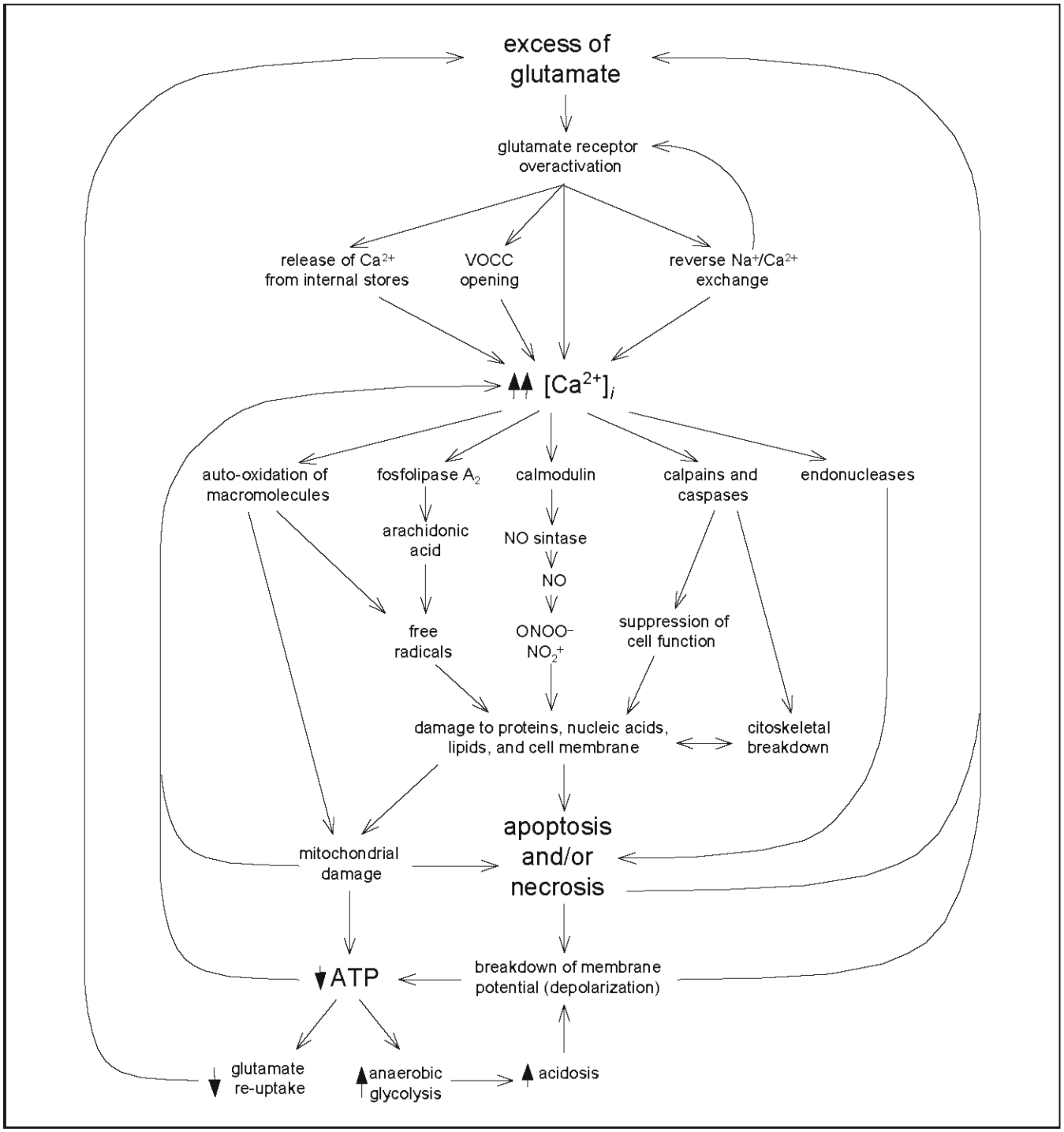


Figure 4: Cellular alterations induced by an excitotoxic lesion. The uncontrolled increase of $[Ca^{2+}]_i$ leads to apoptotic or necrotic neuronal death. Positive feedback of the lesion releases glutamate during this process (Adapted from Tymianski et al. 1996).

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 [51] and helpsmaintain neuronal membrane polarity as a priority. In this situation, our results have shown that intracellular Ca^{2+} may precipitate as hydroxiapatite to reduce its cytoplasmic toxicity as well as the extrusion energy expenses in neurons and astrocytes.

2.3. Brain calcification

2.3.1. Experimental model of brain calcification

Our data show that glutamate analog microinjection in rat CNS leads to intracellular Ca^{2+} precipitation as part of the on-going induced degenerative process [52,72,87], which is similar to brain calcification in humans [53]. We have shown that Ca^{2+} deposits are also induced by blockade of glutamate re-uptake [46]. As these deposits are observed in several areas of rat brain after microinjection of different excitotoxins [7,72,78,87], 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 (Figure 5). For example, sensitivity to AMPA-induced calcification decreased from the globus pallidus, cerebral cortex, hippocampus, medial septum, to retina [72,78]. Moreover, in medial septum, the degeneration associated with microinjection of ibotenic and quisqualic acid was characterized by significant atrophy and no calcification [52,87]. However, in similar conditions, AMPA microinjection resulted in similar atrophy and Ca^{2+} deposits at the injection site.

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 [52,53]. Differences in the neuronal phenotype of either Ca^{2+} buffering and extrusion systems, specific energetic needs, or expression of the glutamate subtype receptor (with a key role of mGluRI) should explain this variability.

The ultrastructural study of the tissue affected by excitotoxicity has also contributed to our understanding of calcification. In basal forebrain and hippocampus we have characterized calcified deposits within hypertrophied astrocytes (Figure 6). They ranged from 0.5 to 10 μm in diameter and were formed by numerous, small, needle-shaped crystals associated with cellular organelles, such as microtubules, cisternae, vesicles or mitochondrias, with no signs of neurodegeneration. Larger inclusions were surrounded by reactive microglia, a finding that was also observed in tissue after specific localization by *in vitro* autoradiography (Figure 7) [7,72,87]. X-ray microanalysis showed an electron-diffraction ring pattern which was characteristic of a crystalline structure similar to apatites [38], and a Ca/P ratio of 1.3±0.2 of cytoplasmic deposits, a ratio lower than the theoretical apatite value of 1.67 (Figure 6). This ratio is also typical of biological crystals which do not have an ideal organization [78]. As biological hydroxyapatites, these deposits are similar to those observed in several peripheral human tissues [32,39].

Experimental models of bone formation (i.e. hydroxiapatite formation *in vitro*) [1] have shown that, instead of Ca^{2+}, a minimal amount of phosphorus, as inorganic phosphate, is crucial for crystal nucleation in a collagen matrix. Similarly, organic phosphate residues of the phosphoproteins also play a direct and significant role in the process of *in vitro* nucleation of apatite by bone collagen, whereas collagen itself does not promote the precipitation of Ca^{2+} and phosphate [1]. Therefore in our experimental model calcification depends on the increase in intracellular inorganic phosphate (i.e. 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.

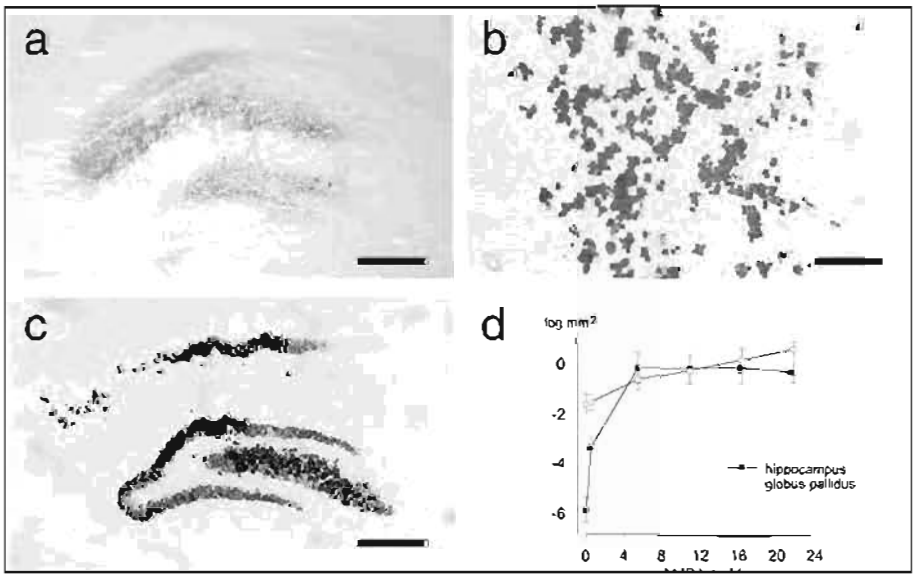


Figure 5 Differences in calcification between rat hippocampus and globus pallidus after AMPA or NMDA microinjections (a) AMPA induces small Ca^{2+} deposits in hippocampus, affecting mainly the CA1 radiatum and lacunosum moleculare layers and the molecular layer of dentate gyrus (b) AMPA microinjection in globus pallidus induces large Ca^{2+} deposits throughout different cerebral areas (c) NMDA infusion in hippocampus induces the formation of large Ca^{2+} deposits, which are mainly located in the pyramidal layer of CA1 and the granular layer of dentate gyrus (d) Comparison of the globus pallidus and hippocampus AMPA dose-response study showed two vulnerabilities to calcification Bars, 300 mm

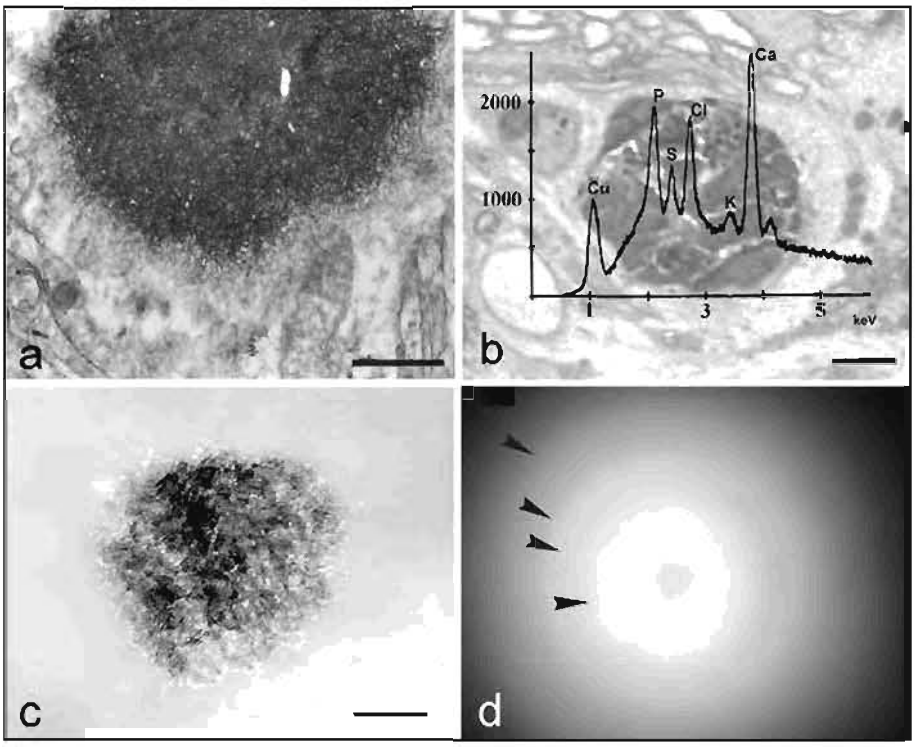


Figure 6 Characterization of Ca^{2+}-deposits (a) Intracellular Ca^{2+} deposit viewed by TEM, note its acicular structure composed by several nanocrystals (b) X-ray microanalysis of a non-osmificated sample with a calculated Ca/P ratio of 1 3 (c) TEM image of a non-osmificated deposit showing needle-shaped 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

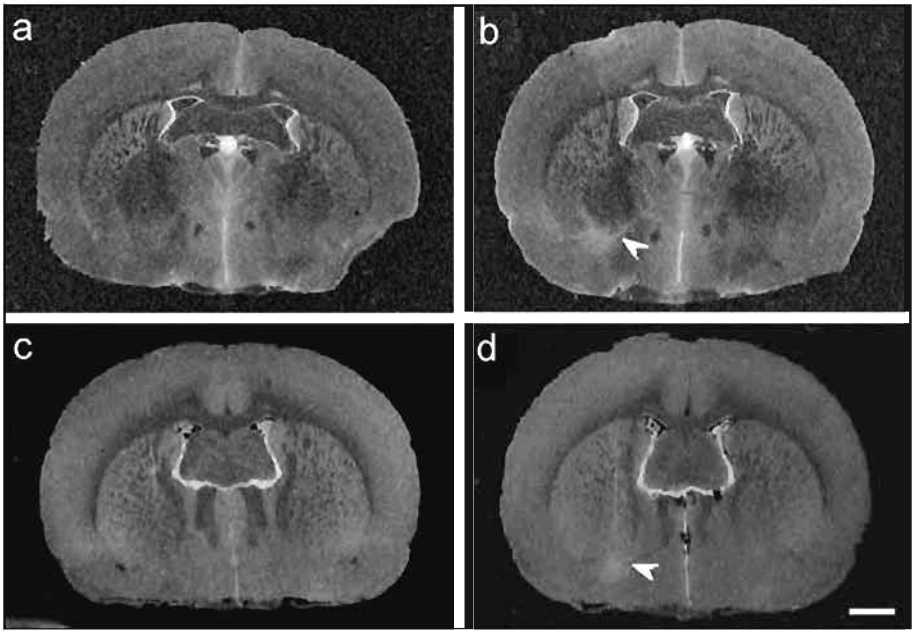


Figure 7 Increase in *in vitro* binding sites of [^{3}H]Iazabemide and [^{3}H]PK11195 as astro– and microglial markers on coronal sections of rat brain after AMPA microinjection (a) [^{3}H]Iazabemide binding showing basal astroglial levels in a control rat (b) AMPA microinjection in globus pallidus induces an increase in [^{3}H]Iazabemide binding (arrowhead), characteristic of an astroglial reaction (c) [^{3}H]PK11195 binding showing basal microglial levels in a control rat, (d) A microglial reaction associated with the AMPA microinjection in globus pallidus is detected as an increase in [^{3}H]PK11195 binding (arrowhead) Bar, 2 mm

In aqueous solutions, hydroxyapatite crystallization takes place in two sequential steps [79]: in the first, crystal nucleation occurs spontaneously with subsequent growth until some nanometers, when phosphate and calcium ions reach a certain concentration; in the second step, an accretion process of these nanocrystals on a proteinic net takes place to reach a maximal size of 20 micrometers. While the first process allows the re-solubilization of the crystal, the second produces a stable precipitate and needs a catalysis agent. These two mechanisms may help explain the size differences we found between several areas of the CNS. For example the large insoluble Ca^{2+} precipitates (mean size 20 μm) after AMPA microinjection in globus pallidus [72] fit well with the second step theory, whereas the small deposits (mean size 3 μm) obtained in hippocampus after the same manipulation [78] may reflect the lack of a catalysis agent for accretion, or an equilibrium between formation and solubilization of crystals. Furthermore, in rat striatum, blockade of glial glutamate uptake [46] produces a spherical lesion with a central necrotic core surrounded by a penumbra zone similar to that caused by focal ischemia. Three days after the treatment, an astroglial reaction and small Ca^{2+} deposits (mean diameter < 1 μm) were observed in the penumbra area. Eleven days later, these deposits had disappeared,

the penumbra zone had recovered from injury and the necrotic area was partially repaired [46]. In this situation, compensatory mechanisms help normalize Ca^{2+} homeostasis and avoid further neuronal death. The tissue recovers the ability to use extrusion mechanisms, and the re-solubilization of Ca^{2+}precipitates takes place.

Together with Ca^{2+} deposits, the excitotoxic lesion induces precipitation of uric acid and aluminosilicates, and the accumulation of sulphated mucosubstances [53]. 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 [104]. Moreover, adenosine inhibits neurotransmitter release and a balance between excitatory and inhibitory neurotransmission may prevent glutamate excitotoxicity [9]. Consequently, the concentration of uric acid increases during neurodegeneration [3] 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 [74] because of the unique affinity of aluminium for silica acid. Precipitates of hydroxyaluminosilicates may therefore easily be 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 [1]. 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 [1]. It should also be noted that, because of their high sulphur content, these mucosubstances may act as antioxidants [53].

2.3.2. Human brain calcification and hypoxia-ischemia

As hypoxic-ischemic injury is a major cause of neurological sequelae through disturbances in Ca^{2+} homeostasis, and premature-neonates are more resistant to hypoxia-ischemia than term neonates, we also studied the relationship between differences in human brain vulnerability to hypoxia-ischemia during the perinatal period and brain calcification in basal ganglia, cerebral cortex, and hippocampus [77]. The number and size of the observed non-arteriosclerotic calcifications were area-specific and increased in term neonates. Basal ganglia presented the highest degree of calcification and hippocampus the lowest, mainly in the CA1 subfield. In all cases, neuronal damage was associated with astroglial reaction and Ca^{2+} precipitates, with microglial reaction absent in hippocampus. These data are consistent with those obtained after long-term excitotoxic lesions in rat brain and support the involvement of EAA receptors in hypoxia-ischemia damage, with a key role of mGluRI. They also suggest a marked similarity of the molecular and cellular mechanisms underlying both processes.

Comparison between lifespan and degree of calcification demonstrated that, in all cases, the highest calcified area was within two months after the hypoxia-ischemia, and that the semi-calcification time was very short (less than 10 days) (Figure 8). This last parameter, independent of subjective

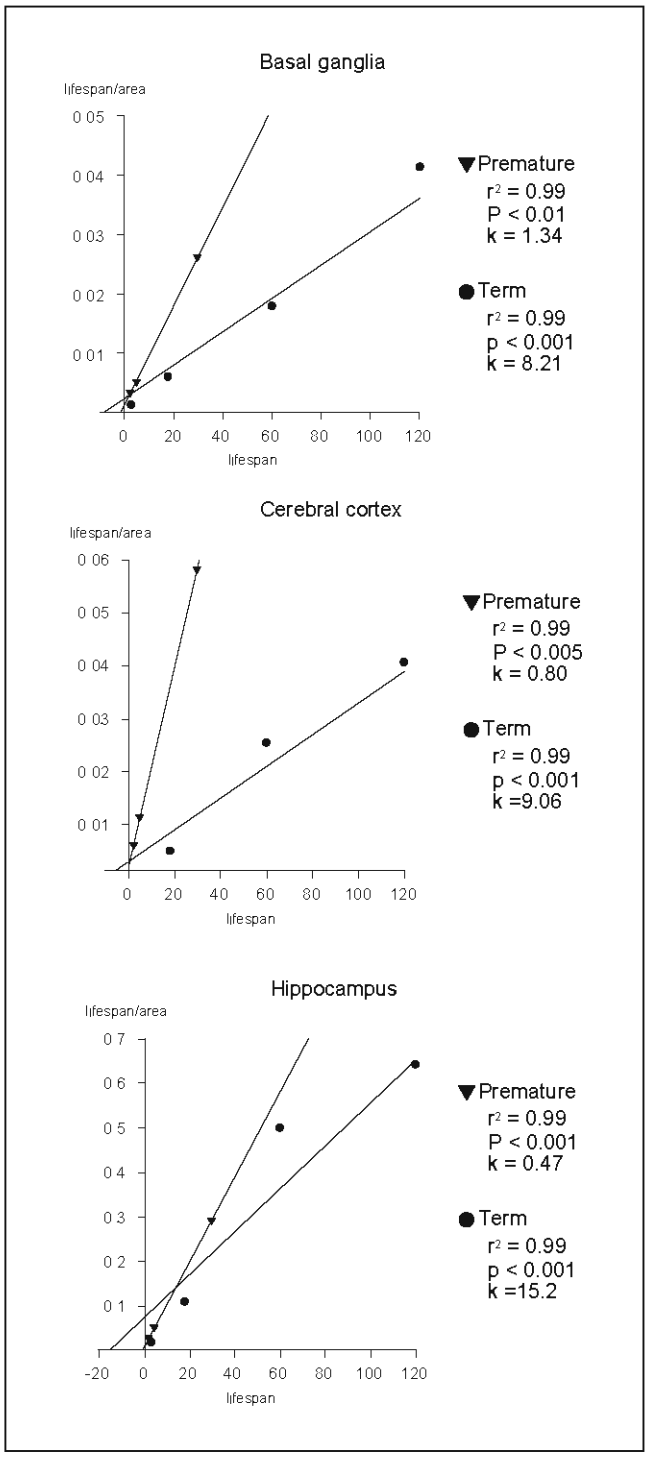


Figure 8: Timing of the hypoxia-ischemia-induced calcification in basal ganglia, cerebral cortex and hippocampus of premature (n = 3) and term (n = 5) human neonates. In the correlation study, the calcified area was calculated in a representative zone (1 mm^2) of each cerebral area, the lifespan corresponds to the time of injury in days. k, days to reach half of the maximal calcified area.

measurements, suggests that calcification depends on the degree of brain differentiation and initial cerebral injury, but not on the time-course of the lesion. Moreover, the mechanisms leading to Ca^{2+} precipitation seem to be similar for all brain areas. If this is true, neurons of each CNS structure degenerate through a common mechanism which is linked to disturbances in Ca^{2+} homeostasis. As each area of the brain participates in specific physiological functions, the resultant pathology will depend on the specific neuronal death of the area affected.

2.3.3. Aging, excitotoxicity and brain calcification

Several studies suggest that aging increases neuronal vulnerability to toxic compounds, including drugs that impair energy metabolism and induce secondary excitotoxic processes [14]. However, a decreased susceptibility of aged rats towards excitotoxins such as quinolinic or kainic acids has been reported [37]. Our data demonstrate that AMPA-induced Ca^{2+} deposits in rat hippocampus are age-dependent since young rats (3 months old) presented greater calcification area than middle-aged ones (15 months old) [6]. In this study, glial reaction, γ-aminobutiric acid (GABA)-uptake activity and immunostaining of Ca^{2+} binding proteins showed the same response. Therefore the vulnerability of hippocampal neurons to AMPA-induced neurodegeneration decreases with age between 3 and 15 months. Similar results have been found in other brain areas such as the striatum and the nucleus basalis magnocellularis. This reduced vulnerability may be related to several factors. For example, age-associated variations in the relative abundance of glutamate receptors and pre-synaptic alterations of glutamate release may explain, at least in part, an increased resistance to excitotoxicity in the hippocampus [60,62].

This effect is compatible with the increased vulnerability to excitotoxicity observed in the eldest animals [14], since some of the factors responsible for the resistance to the insult may follow a biphasic pattern, with a progressive increase until maturity and a later decrease [19]. Many authors have also described a biphasic variation of several parameters during aging, with an opposite tendency before and after middle-age [100]. We observed a biphasic variation of monoamine oxidase B (MAO-B) during aging in most of the brain areas of humans: until the age of 50-60 years MAO-B levels remain unvaried and after start to increase [86]. This observation may be due to the presence of MAO-B rich reactive astrocytes in response to neuronal degeneration. In patients with Alzheimer's disease a similar increase has been found in plaque-associated astrocytes [88]. As MAO-B activity is associated with ROS production, astrocytes may contribute to the age-associated decline of neurological functions. The recent evidence that an increase in AMPA receptor correlates negatively with MAO-B in aged-associated learning-impaired rats also suggests that a gliopathic reaction may be involved in neuronal dysfunction [2].

Table 2.

Neurotransmitter	control (n=15)	13 days (n=5)	21 days (n=5)	30 days (n=5)
Glutamate	100	76 ± 6 *	70 ± 2 *	93 ± 10
Taurine	100	56 ± 3 *	69 ± 16	75 ± 11
Noradrenaline	100	-	-	63 ± 5 *
Octopamine	100	34 ± 3 *	-	-

Long-term effects of the basal forebrain excitotoxic lesion neurotransmitter cortical extracellular levels. Columns are referred to post-lesion time after ibotenic acid injection in basal forebrain. Values show percentage of the control and are expressed as mean ± SEM. * $p < 0.05$ versus control based on the Mann-Whitney U test.

2.3.4. Neurodegeneration, inhibitory neurotransmission and calcification

To counteract excitotoxic processes after excessive glutamate release, homeostatic changes have been reported in different areas of the brain. These changes include the release of inhibitory neurotransmitters, including adenosine and GABA. Thus, adenosine acts as a neuroprotector in hypoxia, ischemia and other situations [82]. Similarly, the simultaneous release of GABA with excitatory amino acids may counteract neuronal cell death during excitotoxicity and ischemia [85]. To clarify the role of these neuromodulators during excitotoxicity, we studied the effects of GABA and adenosine receptor antagonists on NMDA-induced excitotoxicity in hippocampus.

Blockade of adenosine A2a receptors induces an increment in NMDA-induced neuronal death and hippocampal atrophy. This enhanced reduction in the hippocampal area is also observed when a $GABA_A$ receptor antagonist is co-injected with NMDA. Surprisingly, treatment with these antagonists did not significantly modify the magnitude of NMDA-induced Ca^{2+} deposits [75]. Thus, in hippocampus, these inhibitory neurotransmitters may interfere with the NMDA-induced acute excitotoxic lesion by reducing only neuronal death.

2.4. Transynaptic effects of excitotoxicity

One of the consequences of excitotoxic-induced neuronal loss is the alteration of other neurotransmitter systems and neuromodulators. For example, as illustrated in Table 2, long-term ibotenic-induced lesion in the basal forebrain of rat leads to a loss of cholinergic afferences and to decreased extracellular noradrenaline, glutamate, and taurine [9,47]. This cortical reduction in glutamatergic transmission presents a temporal pattern which, with the development of Ca^{2+} deposits and the decrease in the cholinergic and noradrenergic function [87], mimicks the neurochemical modifications described in Alzheimer's disease. Similarly one year after acute lesion, the cortical and hippocampal decrease in brain-derived neurotrophic factor, fibroblastic growth factor,

and glucocorticoid receptor, and the increase in *c-fos* expression in the septal area were still significant [10]. Thus, excitotoxic lesions in basal forebrain can modify long-term cortical adaptative responses, and may modulate the expression of glutamate receptor. Some of these effects, such as the decrease in brain-derived neurotrophic factor and the increase in *c-fos* expression, also reflect the molecular alterations present in Alzheimer's disease.

Our experimental model may also help to explain this neurological disorder. We showed a dramatic decrease in cortical extracellular octopamine [4], a biogenic amine which, in rodents, decreases with age and is involved in the control of cognitive functions. The lack of data in human tissue opens a new line of study to investigate whether octopamine constitutes a marker of age-associated neurodegeneration in humans.

2.5. Iintracellular calcium increase and energy failure

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

2.5.1. Acidosis

Excitotoxicity induces acidosis in cells and in the extracellular space [31]. 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 [90]. Furthermore, H^+ also appears during some chemical reactions such as phospholipid hydrolysis. In parallel, Ca^{2+} influx causes rapid cytoplasmic acidification [31,102] 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 [96].

Although the mechanisms by which acidosis produces neuronal damage remain unclear, some hypotheses are proposed. H^+ may reduce K^+ conductance and thus facilitate action potentials [50]. Moreover, reinforced by energetic depletion, a decrease in pH inhibits the Na^+/Ca^{2+} exchange, thereby contributing to the breakdown of membrane potential and increasing, again, the $[Ca^{2+}]_i$ (Figure 4). Acidosis may also inhibit neurotransmitter re-uptake, enhance free radical production or accelerate DNA damage [96]. However, a pH decrease helps prevent further neuronal damage by NMDA receptor blockade and Ca^{2+} influx reduction into the cell [29,36].

2.5.2. Reactive oxygen species production

ROS are molecules, ions or atoms with one unpaired electron in their most external engaged orbital, which makes them highly reactive. In normal conditions, low concentrations of ROS are produced during cellular processes such as mitochondrial electronic transport and some enzymatic activities (e.g. MAOs, tyrosine hydroxilase, and xantine oxidase). ROS may be involved in the modulation of some physiological functions like the regulation of neuronal excitability [103]. In neurons, stimulation of NMDA receptor induces the activation of the Ca^{2+}-dependent phospholipase A_2 with the subsequent production of arachidonic acid (Figure 2), which controls a phospholipidic metabolic pathway that is involved in the production of ROS [20]. These species can be eliminated by two antioxidant mechanisms: a) three molecules, namely ascorbic acid, vitamin E and glutathion, participate in reducing cellular ROS ; and b) three enzymes degrade ROS activity in the brain, superoxide dismutase, quinone reductase and, the most abundant, astroglial glutathion peroxidase.

During hypoxic injury, the reduced flavin adenine dinucleotide and coenzyme Q auto-oxidize to produce ROS because of insufficient O_2 availability [96]. As a consequence, cytochrome *c*, which is normally confined to the mitochondrial intermembrane space, is released into the cytosol [41], decreasing the cellular respiratory capacity even further. Moreover, over-stimulation of NMDA receptors increases eicosanoid metabolism, which contributes to the uncontrolled increase in neuronal ROS [96].

Studies of ischemia and reperfusion have shown that high $[Ca^{2+}]_i$ can activate a Ca^{2+}-dependent protease which catalyzes the xantine dehydrogenase conversion to xantine oxidase. Ischemia also induces ATP degradation to hypoxantine, a substrate of xantine oxidase; O_2 provided during reperfusion is the other substrate of the reaction. Consequently, xantine oxidase is strongly activated and produces large amounts of uric acid and ROS. Free radicals interact with phospholipids, proteins, nucleic acids, glycosaminoglycans (Figure 4) and, specially with amino acids that contains sulfide and unsaturated fatty acids [20].

2.5.3. Protease activation, apoptosis and necrosis

Proteases of the caspase and calpaine families have been implicated in neurodegeneration, as their activation can be triggered by Ca^{2+} influx and oxidative stress (Figures 4 and 9). 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 [96]. 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 [55].

The dysregulation of neuronal Ca^{2+} homeostasis during acute ischemic insults, epileptic seizures, and traumatic brain injury may result in excessive stimulation of calpains

(See Section 1.4) [83]. 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 [16] (Figure 9).

Calpain is activated in most forms of necrosis and in some forms of apoptosis, while caspase 3 is only activated in neuronal apoptosis [101]. 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 cysteine proteases, but do not require Ca^{2+} for activity [101], although they are also responsive to increase intracellular concentration of this ion[16].

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 [101]. 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. [16]. 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 recently been described [63]. 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 [81]; the apoptotic death being associated with the 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 [81].

2.6. Our hypothesis on brain calcification

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

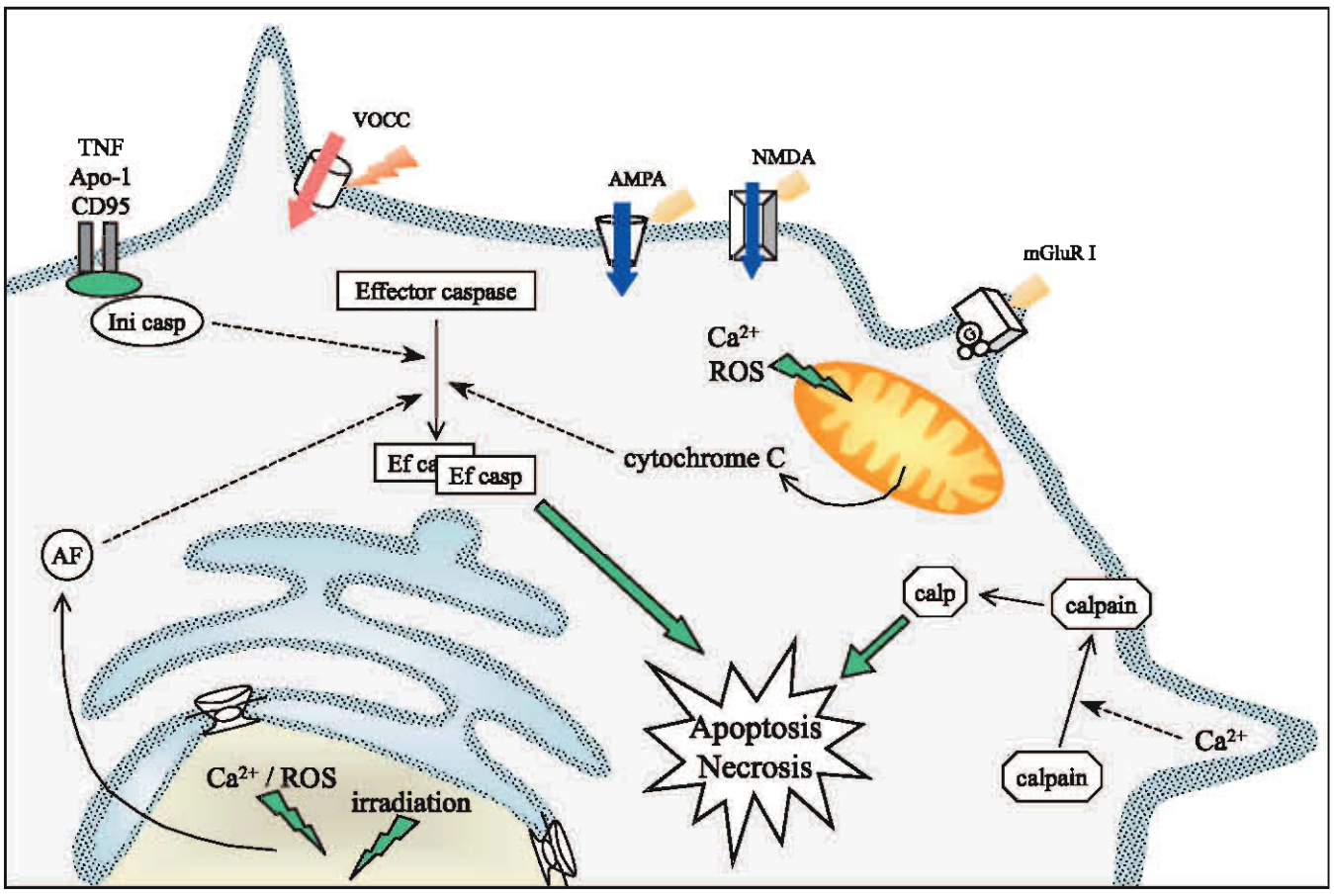


Figure 9: Pathways for protease activation. Calpains are activated when calcium increases in the cell. There are at least two major pathways by which the initiator pro-caspases (Ini casp) are activated in response to death-inducing stimuli, and subsequently cleave the effector caspases: one involves death receptors such as TNF and CD95, the other is a receptor independent process that consists of apoptotic signals such as calcium or ROS. Once activated, the death receptors recruit initiator caspases for their proteolytic activation. The apoptotic signals may act on the mitochondria to induce the release of cytochrome *c*, or on the nucleus to release unknown apoptogenic factors (AF). (Adapted from Chan et al. 1999)

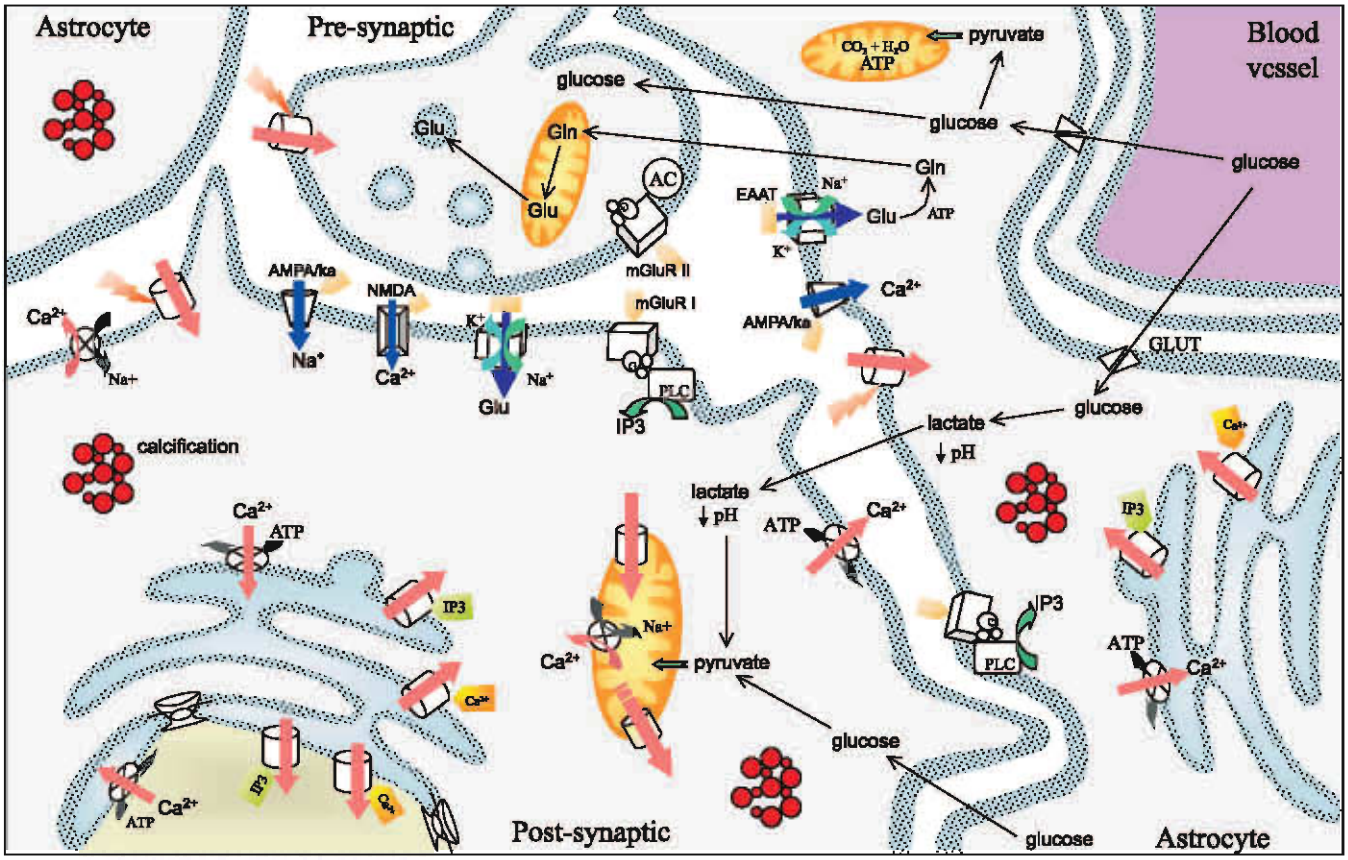


Figure 10: Schematic drawing of the excitotoxic process induced by glutamate with the intracellular step of precipitation as part of Ca^{2+} homeostasis. The metabolic pathway of lactate with the communication between the endothelial, glial and neuronal compartments is included in the diagram

of Ca^{2+} and the formation of $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-} ions from inorganic phosphate [23] 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. 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.

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 [53,78], despite the fact that mitochondrial dysfunction constitutes a primary event in NMDA-induced degeneration in cultured hippocampal neurons [89]. 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 [93].

Other results support this hypothesis. In rat globus pallidus, the AMPA-dose-response study between 0.54 and 21.6 mM has shown an increase in calcification which was not accompanied by an increase in astrogliosis [72]. In hippocampus, 2.7 mM AMPA induced a calcified area larger than the injured area [5]. In this same structure, the selective adenosine-A2a-receptor antagonist 8-(3-chlorostyryl)-caffeine increased the NMDA-induced neuronal loss while calcification was decreased [75]. Thus, all these data indicate that Ca^{2+} precipitation does not necessarily reflect neuronal death. They may also indicate that, as proposed for retinal excitotoxic damage [17], besides Ca^{2+}, other factors such as Na^+ and Cl^- influx, K^+ efflux and swelling induce excitotoxic neuronal damage.

Finally, several CNS disorders can be induced after the same injury because of differences in neuronal populations and abundance and distribution of glutamate receptor subtypes. This variability is shown by the appearance of distinct neurodegenerative parameters and it determines the induction of a chronic process. Thus at the tissue level, the response against the initial injury is compensated or produces various lesions depending on 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 mGluR 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.

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About the authors

Since the mid 90s, the researchers of the Grup de Neuroquímica of the Facultat de Medicina, IDIBAPS, of the Universitat de Barcelona have conducted research into a simplified model of neurodegeneration of the central nervous system in rats to better understand the cellular and molecular mechanisms underlying neurodegenerative processes in humans.

Stereotaxic microinjections of EAA receptor analogs have been used to induce an excitotoxic lesion in several areas of the rat brain and, afterwards, the temporal and spatial evolution of the lesion has been boarded from morphological, biochemical and pharmacological points. For example, in vivo intracerebral microdialysis allows the monitoring of modifications in synaptic trafficking in specific nuclei and their related brain areas, where functional and compensatory responses were also observed. Adaptative changes of the genetic expression have been studied by in situ hibridization and, most recently, by differential display reverse transcriptase-polymerase chain reaction (DDRT-PCR). Moreover, at the protein level, immunohistochemical and in vitro autoradiographic procedures have been performed to study variations in monoamine oxidase activity, Ca^{2+} binding proteins, or neurotransmitter receptors; the relevance of the astro– and microglial participation in each neurodegenerative process has also been characterized. Pharmacological studies were undertaken to validate some proposed treatments. Finally, transmission electron microscopy (TEM) and X-ray microanalysis have been performed for ultrastructural studies.

We have also examined the formation of Ca^{2+} deposits as a new aspect of Ca^{2+} homeostasis linked to the neuronal lesion produced in our animal models and in human hypoxia-ischemia, aging or Alzheimer's disease; and we showed that reduction of both Ca^{2+} precipitates and neuronal death occurs simultaneaously. We are currently working on the identification of the proteins involved in these mechanisms.