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Intermediate Filaments as a Target of Signaling Mechanisms in Neurotoxicity

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Abstract

In this chapter, we deal with the current knowledge and important results on the cytoskeletal proteins and their differential regulation by kinases/phosphatases and Ca^{2+}-mediated mechanisms in developmental rat brain. We focus on the misregulation of the phosphorylating system associated with intermediate filament proteins of neural cells and its relevance to cell and tissue dysfunction. Taking into account our findings, we propose that intermediate-filament proteins are dynamic structures whose regulation is crucial for proper neural cell function. Given their relevance, they must be regulated in response to extracellular and intracellular signals. The complexity and connection between signaling pathways regulating intermediate-filament dynamics remain obscure. In this chapter, we get light into some kinase/phosphatase cascades downstream of membrane receptors disrupting the dynamics of intermediate filaments and its association with neural dysfunction. However, intermediate filaments do not act individually into the neural cells. Our results evidence the importance of misregulated cytoskeletal crosstalk in disrupting cytoskeletal dynamics and cell morphology underlying neural dysfunction in experimental conditions mimicking metabolic diseases and nongenomic actions of thyroid hormones and as an end point in the neurotoxicity of organic tellurium.

Keywords: intermediate filament, cytoskeleton, cell signaling, calcium, neurotoxicity

1. Introduction

All the cell functions accomplished by the living cell are dependent on a sophisticated network of protein filaments with different compositions, distributions and roles into the cell, forming an integrated meshwork known as the cytoskeleton. However, the most striking feature of the cytoskeleton concerns its ability to respond to signals and conditions to which cells are submitted, taking part of adaptive cell response to different stimuli. The cytoskeleton is

an end point of signaling pathways adapting cells to immediate or long-lasting behaviors in healthy and sick organisms.

Cytoskeleton of most animal cells is constituted by three interconnected filament subsystems: microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs). Compelling evidence from the last decades has brought convincing understanding for the highly regulated and interconnected interactions between the cytoskeletal elements giving support to sculpting and maintaining cell shape and sustaining all kinds of morphological alterations or internal organization, as well as their implications for the behavior of animal cells. **Figure 1** demonstrates the organization of the cytoskeleton in neurons.

A cohort of accessory proteins and signaling machinery regulates the dynamic turnover of the cytoskeleton. Although each type of filament has specific cell distribution, molecular constituents and equilibrium, the coordinated intertwining among the different networks provides the force for a number of coherent processes in response to all kinds of intra- and extracellular stimuli leading responses so decisive as cell survival or death [1].

This chapter initiates with a brief introduction about the structure and function of IFs, emphasizing those from neural cells. However, the main purposes of the chapter are the experimental evidence of our laboratory that the roles of IFs are beyond protection from mechanical and nonmechanical stress. They might be the end point of misregulated-signaling mechanisms in neurotoxic conditions adapting their dynamics, in concert with the other cytoskeletal fibers, to cell survival or death.
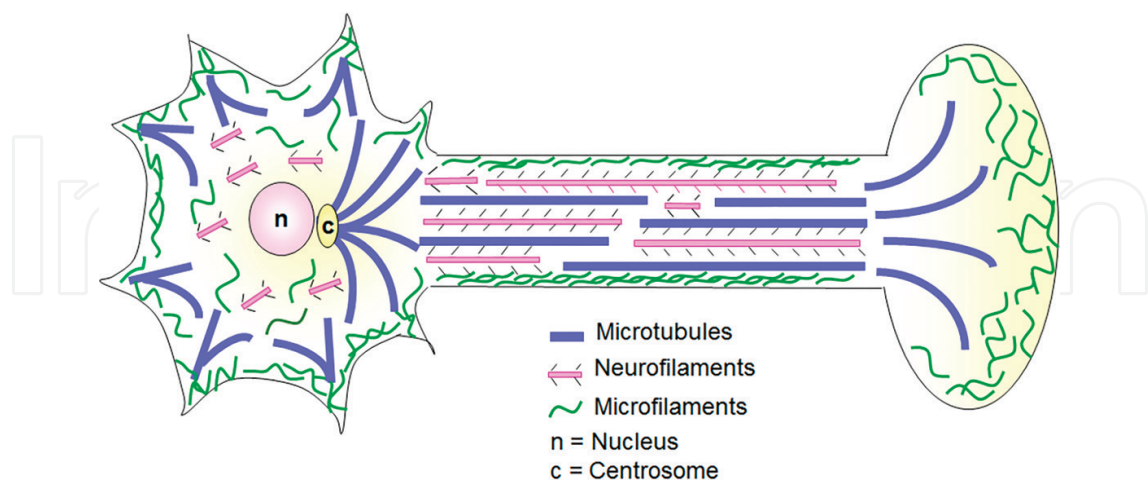

Figure 1. Distribution of cytoskeletal constituents into neurons. Neuronal cytoskeleton is composed by microfilaments, microtubules, and intermediate filaments. The microtubules are nucleated at the centrosome, then released and delivered to either the dendrites or the axon. Neurofilaments are abundant in axons and the spacing of neurofilaments is sensitive to the level of phosphorylation. The microfilaments are dispersed within the cells and they are most abundant near the plasma membrane.

2. Intermediate filaments

2.1. Molecular architecture of intermediate filaments

IFs are flexible, rod-shaped fibers averaging 10 nm in diameter, a size that is intermediate between MFs and MTs. They are ubiquitous constituents of the structural scaffold of the eukaryotic cells and considered mechanical integrators of cytomatrix [2]. These cytoskeletal filaments are widespread expressed in practically all animal cell types and are the most diverse cytoskeletal protein family, encoded by an estimated 70 IF genes in the humans. IFs have been grouped into six sequence homology classes (SHC) according to the degree of sequence identity: acidic keratins (SHC group I); basic keratins (SHC group II); desmin, vimentin and other mesenchymal IF proteins, such as glial fibrillary acidic protein (GFAP) (SHC group III); neurofilament proteins (SHC group IV); and lamins (SHC group V).

IF building blocks are fibrous proteins stabilized by multistranded left-handed coiled coils giving rise to a rope-like structure. Their structures are constituted by a long central α-helical region, also designed rod domain, with a distinct number of equally sized coiled coils forming segments flanked by non-α-helical N-terminal (the head domain) and C-terminal domains (the tail domain). Both head and tail domains are highly varying in size and sequence, thus, the functional and molecular heterogeneity of IF proteins are a consequence of the highly variable non-α-helical end domains of subunits.

The central rod domain of IF subunits is α-helical rod highly charged, with a role in the first phase of IF assembly. By contrast, the head domain enriched in basic amino acids is essential for the formation of tetramers (the polymerization units) and complete IF assembly.

The non-α-helical tail domain can vary drastically between different IF proteins. This domain is not essential for the assembly of cytoplasmic IFs but plays a significant role in filament width control. The functional role of the tail domain is particularly important in the neurofilaments, the neuronal-specific IFs, as discussed below.

Overall, the assembly of subunits giving rise to functional IFs is a complex and multistep process with individual specificities among the different representatives of this molecularly heterogeneous family. Taking into account the *in vitro* polymerization of vimentin, filament assembly starts with the formation of parallel, in-register dimers. These dimers spontaneously associate laterally into antiparallel, half-staggered tetramers. Tetramers aggregate into higher-order oligomers to form unit length filaments (ULFs) that undergo reorganization and elongation by longitudinal annealing to form immature IFs. The final step is radial compaction of the filaments from approximately16 nm to a diameter of 10–12 nm [3].

Different from the other IFs, NFs comprise three subunits with different molecular masses and distributions into the filament. They are formed by light, medium and heavy molecular mass NF triplet proteins (NF-L, NF-M and NF-H), respectively. NF-L can self-assemble forming the core of the filament. NF-M and NF-H are peripherally disposed on the filament, with their long and flexible tails rich in highly charged domains and multiple phosphorylation sites, radially projecting out from the filament backbone when NF-M and NF-H co-assemble

with the short-tail NF protein NF-L. Interestingly, NF-H and NF-M by their own are not able to assemble into filaments, but by contrast, self-assembled NF-L yields normal looking 10-nm filaments. These side arms of NF-M and NF-H contain multiple phosphorylation sites regulating the interactions of NFs with each other and with other cytoskeletal structures [4].

2.2. Roles of intermediate filaments in neural cell function

Neurons are highly specialized in the transmission and processing of electrical and chemical signals. A functional nervous system is dependent of a proper axonal array, which in turn is critically dependent upon the organization of the axonal cytoskeleton. Five main subunit proteins form the neuronal specific NFs: the group IV NF-L, NF-M and NF-H triplet proteins, α-internexin and the group III peripherin. Mature filaments are composed of several combinations of these five subunits. In most differentiated neurons, α-internexin expression precedes that of the NF triplet and declines somewhat postnatally, while the expression of the NF triplet sharply rises. Neurofilaments found in perikarya, dendrites and axons differ considerably in their organization and function. Perikarial NFs form a meshwork around the nucleus. In the axons of mature neurons, a large number of longitudinally oriented and phosphorylated NFs play a fundamental role increasing the diameter of myelinated axons and consequently nerve conductivity. Neurofilaments present in dendrites are less abundant and less phosphorylated than those of axons.

Neurofilaments are transported from the cell body, where they are synthesized, to be delivered along the axon by a mechanism called axonal transport. The motors implicated in the anterograde transport are kinesins, while the retrograde transport is mediated in association with dynein, the same motor proteins involved in the fast axonal transport along MTs [4].

The multiple roles of cytoskeletal proteins in the neural cells imply that there is an underlying cytoskeletal pathology associated with several neurodegenerative processes. The major neurodegenerative diseases are characterized by the presence of inclusion bodies in implicated neurons. These inclusion bodies all contain elements of the cytoskeleton. In addition, mutations and/or accumulations of NFs are frequently observed in several human neurodegenerative disorders including amyotrophic lateral sclerosis, Alzheimer disease, Parkinson disease, Charcot-Marie-Tooth, giant axonal neuropathy, neuronal intermediate-filament inclusion disease and diabetic neuropathy [5]. Multiple factors can potentially induce the accumulation of NF, including deregulation of NF gene expression, NF mutations, defective axonal transport, abnormal posttranslational modifications and proteolysis [4]. Beyond their association with neural damage in inherited or age-dependent neurodegenerative diseases, studies from our laboratory indicated that the disruption of NF homeostasis is a response to toxic agents and abnormally accumulated metabolites in rat brain.

Astrocytes are important cytoarchitectural elements of the CNS; however, during the past few years, molecular and functional characterization of astroglial cells indicates that they have a much broader function than only support the neurons in the brain. Compelling evidence supports that astrocytes have specialized functions in inducing and regulating the blood-brain barrier (BBB), glutamate uptake, synaptic transmission, plasticity and metabolic homeostasis of the brain [6]. Astrocytes express 10 different isoforms of glial fibrillary acidic protein

(GFAP), the specific astrocytic IF, together with vimentin, nestin and synemin. However, GFAP is the main IF protein expressed in mature astrocytes, where it helps maintaining mechanical strength, as well as cell shape. However, recent evidence has shown that GFAP plays a role in a variety of additional astrocyte functions, such as cell motility/migration, cell proliferation, glutamate homeostasis, neurite outgrowth and injury/protection [7].

Astrocytes are also involved in a wide range of CNS pathologies, including trauma, ischemia and neurodegeneration. In such situations, the cells change both their morphology and their expression of many genes leading to activation of astroglia, or astrogliosis. It is accepted that the increase of IFs with accompanying cellular hypertrophy and an abnormal apparent increase in the number of astrocytes characterize astrogliosis. However, upregulation of IF proteins, in particular GFAP, but also vimentin and nestin, two IF proteins abundantly expressed in immature astrocytes, is regarded as the hallmark of astrogliosis [7]. In this regard, the most remarkable evidence of the relevance of GFAP in the physiological roles of astrocytes in maintaining normal brain function is Alexander disease, a fatal disorder in which GFAP mutations might compromise the astrocyte stress response [8].

3. Protein phosphorylation in signaling transduction

Phosphorylation is the most widespread type of posttranslational modification of the intracellular signaling proteins. Phosphorylation of proteins occurs within seconds or minutes of a regulatory signal, typically an extracellular signal.

Phosphorylation is an enzymatic process in which the introduction of a phosphoryl group to specific amino acid residues of a protein is catalyzed by protein kinases and the removal of phosphoryl groups is catalyzed by protein phosphatases. For phosphorylation to be useful in the regulation of a protein activity, it is important to be a reversible process, in which the phosphorylated form of the protein could restore its original dephosphorylated form when signal ends, functioning therefore as a molecular switch. The addition of a phosphoryl group to the side chain of a Ser, Thr, or Tyr residue introduces a bulky, charged group into a polar region. The oxygen atoms of a phosphoryl group can hydrogen bond with one or several groups in a protein, commonly the amide groups of the peptide backbone at the α-helix start or the charged guanidinium group of an Arg residue influencing the functionality of the protein [9].

3.1. Phosphorylation of intermediate-filament proteins

Phosphorylation, glycosylation and transglutamination take part in the multiple mechanisms of IF regulation. However, phosphorylation/dephosphorylation is a major regulatory mechanism orchestrating IF dynamics. Phosphorylation sites of IF subunits are located on their head and tail domains and phosphorylation plays a major role in regulating the structural organization and function of these cytoskeletal proteins in a cell- and tissue-specific manner [10].

Amino-terminal phosphorylation regulates the assembly/disassembly equilibrium of type III and IV IFs. Second messenger-dependent protein kinases add phosphate groups on the

amino-terminal head domain on GFAP, vimentin and NF-L. Specific phosphorylating sites for cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (PKCaMII) and protein kinase C (PKC) are associated with IF disassembly; however, the action of the protein phosphatases 1, 2A and 2B (PP1, PP2A and PP2B), respectively, removes phosphate and restores the IF ability to polymerize [11].

Otherwise, the main phosphorylation sites on NF-M and NF-H are located in Lys-Ser-Pro (KSP) repeat regions of the tail domain of these subunits. The KSP repeats are phosphorylated by proline-directed kinases such as Cdk5, the mitogen-activated protein kinases (MAPK) such as Erk1/2, JNK, p38MAPK as well as glycogen synthase kinase 3 (GSK3). Phosphorylation of these KSP sites regulates the interactions of NFs with each other and with other cytoskeletal structures, since the tail domain of NF-M and NF-H protrudes laterally from the filament backbone to form "side-arms" when phosphorylated. These lateral interactions are central in the formation of a cytoskeletal lattice that supports the mature axon. Moreover, carboxyl-terminal phosphorylation of NF-M and NF-H subunits has long been considered to regulate their axonal transport rate and in doing so to provide stability to mature axons [12]. The axonal transport of NFs results from binding to the fast motor proteins kinesin and dynein intermitted with prolonged pauses. It is known that carboxyl-terminal phosphorylation of NF-H progressively restricts the association of NFs with kinesin and stimulates its interaction with dynein. This event could represent one of the mechanisms by which aberrant carboxyl-terminal phosphorylation would slow NF axonal transport. Both the maintenance of axonal caliber and axonal transport are dependent on the adequately phosphorylated NF subunits. Consequently, abnormally hyperphosphorylated NF subunits, commonly found in several neurodegenerative diseases, are intimately associated with neural dysfunction and considered a hallmark of neurodegeneration. In addition, demyelinating diseases might be associated with hypophosphorylated NFs, compromised axonal transport and decreased axonal diameter, since the phosphorylation of NFs occurs in close proximity to myelin sheaths, which release signals needed to induce phosphorylation of NFs in mature axons [13].

In the next sections, we discuss the recent findings from our laboratory indicating that signaling mechanisms involved in the regulation of IF phosphorylation/dephosphorylation are important targets of neurotoxins, metabolites accumulating in neurodegenerative diseases as well as thyroid hormones, emphasizing the relevance of cytoskeletal homeostasis on the brain function/dysfunction. To assess the effects of the neurotoxicants on the phosphorylation level of IF proteins, we developed an approach to measure the *in vitro* incorporation of radioactive phosphate (^{32}P-orthophosphate) into these proteins [14]. In order to shed light onto the signaling cascades targeted by them, we used pharmacological and immunological approaches, specific enzyme inhibitors, channel blockers, or glutamate antagonists as well as monoclonal antibodies directed to signaling cascades or specific phosphorylation sites. We conclude that misregulated cell signal transduction interferes with the phosphorylation/dephosphorylation of IFs disrupting the homeostasis of the cytoskeleton of astrocytes and neurons and this is associated with cell dysfunction and neurodegeneration in experimental models of neurotoxicity. **Figure 2** corresponds to a schematic representation of the consequences of misregulated NF phosphorylation for neuronal function.
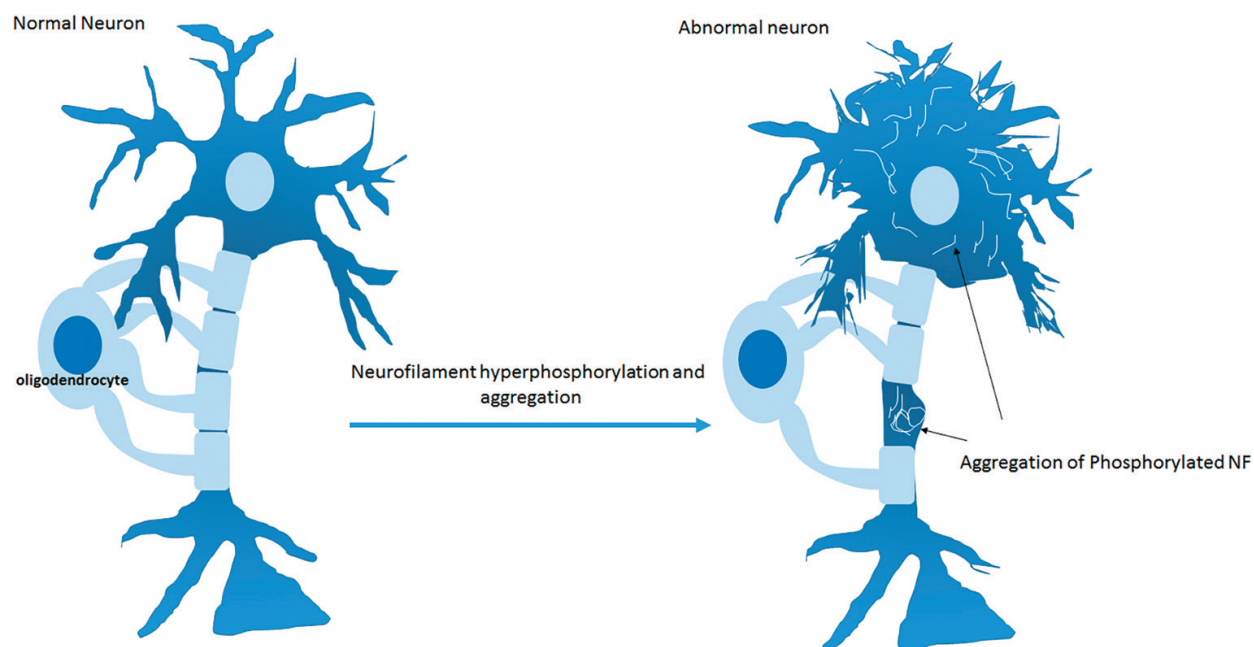
Figure 2. Schematic representation of disrupted neurofilament phosphorylation. The hyperphosphorylation of neurofilaments can change the cytoskeleton architecture and lead to neurofilament aggregation in perikarya and in axon accounting for cell damage.

3.2. Central roles of Ca²⁺ and glutamate receptors on the regulation of cytoskeletal dynamics in neural cells

Changes in the cytoplasmic free Ca^{2+} concentration constitute one of the main pathways by which information is transferred from extracellular signals received by animal cells to intracellular sites. However, an augmented Ca^{2+} influx through the NMDA receptor or voltage-dependent calcium channels (VDCCs) can be responsible for the activation of lethal metabolic pathways in neural cells. Overactivation of glutamate receptors produces neuronal membrane depolarization. This causes the influx of Ca^{2+} into the cytoplasm and subsequently triggers cascade events leading to excitotoxic neuronal death. Excitotoxicity is recognized as a major pathological process of neuronal death in neurodegenerative diseases involving the CNS. In this regard, compelling findings point to the cytoskeleton as an end point of excitotoxic mechanisms.

Different toxins and stress conditions are implicated in the misregulation of intracellular Ca^{2+}-dependent processes in cells and different cell types exhibit a diverse range of transient responses to their stimuli. Exposure of tissue slices to neurotoxicants or metabolites in toxic concentrations triggers the activation of ionotropic and metabotropic glutamate receptors as well as L-VDCC and the endoplasmic reticulum (ER) Ca^{2+} channels. These receptors and channels activate several intracellular-signaling complexes altering cell behavior in a spatio-temporally regulated manner. Metabolism of cyclic nucleotides, membrane phospholipids as well as endogenous enzymatic regulators are the key biochemical steps coordinating cell response to an extracellular stimulus [15].

Calcium is a critical regulator of cytoskeletal dynamics. Dysregulation of Ca^{2+} homeostasis is an important event in driving the disruption of assembly/disassembly equilibrium as

well as the interaction of cytoskeletal proteins with regulatory proteins or cell organelles. In particular, IF proteins are directly regulated by Ca^{2+} levels, which crosslink signaling cascades and connect physiological or pathological extracellular signals with the IF cytoskeleton influencing multiple aspects of cell behavior. Consequently, abnormally elicited Ca^{2+} signals provoking misregulation of key phosphorylation cascades are able to disrupt cytoskeletal homeostasis and this is commonly associated with the cell damage.

4. Toxicity of diphenyl ditelluride on the cytoskeleton of neural cells

Many processes in the organic synthesis, vulcanization of rubber and in metal-oxidizing solutions to tarnish metals, such as silver, extensively use tellurium. Diphenyl ditelluride $(PhTe)_2$ is the simplest of the aromatic, diorganoyl ditelluride compounds used in organic synthesis. Indeed, developmental exposure to $(PhTe)_2$ is teratogenic and is associated with long-term behavioral and neurochemical changes in rats. Until recently, the general toxicity of $(PhTe)_2$ was considered to be exclusively related to the oxidation of thiol-containing proteins (for review, see [16]). However, compelling evidence from our laboratory points to an important role played by signaling mechanisms involved in regulating IF phosphorylation/dephosphorylation as target of $(PhTe)_2$ neurotoxicity. In addition, we evidence a remarkable role of Ca^{2+} mediating these actions secondary to glutamate receptors and L-VDCC activation.

The neurotoxicity of $(PhTe)_2$ is spatiotemporally regulated, consistent with the window of susceptibility of signaling cascades as well as the structural and functional heterogeneity of neurons in different brain regions. In this regard, exposure of cortical slices from 18- and 21-day-old rats to $(PhTe)_2$ shows unaltered phosphorylation of IF proteins, while IFs of acute cortical slices from younger pups (9 and 15 days old) are hypophosphorylated. Activated ionotropic glutamate receptors, L-VDCC and ryanodine channels result in PP1-mediated hypophosphorylation of GFAP and NF subunits pointing to the cortical cytoskeleton as a preferential target of the action of phosphatases in this window of vulnerability. Activation of PP1 is modulated by dopamine and cyclic AMP-regulated neuronal phosphoprotein 32 (DARPP-32), an important endogenous Ca^{2+}-mediated inhibitor of PP1 activity. Depending on the site of phosphorylation, DARPP-32 is able to produce opposing biochemical effects, that is, inhibition of PP1 activity or inhibition of protein kinase A (PKA) activity [17]. Decreased cAMP and PKA catalytic subunits support that $(PhTe)_2$ disrupts the cytoskeletal-associated phosphorylating/dephosphorylating system of neurons and astrocytes through PKA-mediated inactivation of DARPP-32, promoting PP1 release and hypophosphorylation of IF proteins of those neural cells [18]. Regarding neurons, hypophosphorylation of IF proteins could be associated with cell dysfunction since decreased phosphorylation of KSP repeats in the carboxyl-terminal domains of NF-M and NF-H correlates with impaired axonal transport and increased NF-packing density.

In contrast with younger rats, hippocampal slices of 21-day-old rats acutely exposed to $(PhTe)_2$ result in hyperphosphorylated IFs. Hippocampal IF hyperphosphorylation is partially dependent on L-VDCC, NMDA and ER Ca^{2+} channels. The signal evoked by $(PhTe)_2$ is also transduced through metabotropic glutamate receptors on the plasma membrane, leading to the activation of phospholipase C (PLC) that produces the intracellular messengers inositol

1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to specific receptors on the ER changing the conformation of IP3 receptors and opening the channel. Released Ca^{2+} and DAG directly activate PKCaMII and PCK, resulting in the hyperphosphorylation of some of the critical amino acid residues in the carboxyl-terminal tail domain of NF-L known to interfere with filament assembly. In addition, the activation of Erk1/2 and p38MAPK results in hyperphosphorylation of KSP repeats of NFM. Interestingly, PKCaMII and PKC are upstream of MAPK activation implying in a significant cross-talk among signaling pathways elicited by $(PhTe)_2$ that connect the glutamate metabotropic cascade with the activation of Ca^{2+} channels. The final molecular result is the extensive phosphorylation of amino- and carboxyl-terminal sites on IF proteins and deregulated cytoskeletal homeostasis [19].

4.1. Diphenyl ditelluride disrupts the cytoskeleton and provokes neurodegeneration in acutely injected young rats

The *in vivo* exposure to $(PhTe)_2$, in which the neurotoxicant reaches the brain via systemic circulation, also results in different susceptibilities of the IF proteins from neural cells. This can be evidenced in cerebral cortex and hippocampus of 15-day-old rats acutely injected with a toxic dose of $(PhTe)_2$ (0.3 µmol/kg body weight) [20]. Cortical hyperphosphorylation of neuronal and glial IF proteins is an early and persistent event up to 6 days after injection, accompanied by increased levels of GFAP and NF-L. Upregulated gene expression as well as GFAP and vimentin hyperphosphorylation could be a response to injury and take part in the program of reactive astrogliosis, as further demonstrated in striatum [21] and cerebellum [22] of $(PhTe)_2$-injected rats. In addition, hippocampal IFs are not responsive to the insult until weaning. A strong evidence supports an important role of astrocytes in a more severe cortical than hippocampal damage following the *in vivo* $(PhTe)_2$ insult. This supports a direct action of the neurotoxicant on intracellular signaling pathways and highlights the relevance of the interplay between glial and neuronal cells to adapt the cellular metabolic response to the insult even when the brain connections are only partially preserved, as shown in acute brain slices.

Of importance, neurodegeneration is part of the deleterious *in vivo* effects of $(PhTe)_2$ toxicity, as demonstrated in the striatum [23] and cerebellum [22] of $(PhTe)_2$-injected rats. Neurodegeneration is associated with alterations in Ca^{2+} homeostasis and glutamatergic neurotransmission, upstream of inhibited Akt and activated caspase 3. We therefore propose that excitotoxicity is a main mechanism of neurodegeneration caused by this compound in the developing rat brain. On the other hand, most of the actions of $(PhTe)_2$ disrupting the homeostasis of the cytoskeleton in neural cells are mediated by high Ca^{2+} levels. Moreover, a link among disrupted IF homeostasis, activated astrocytes and neuronal apoptosis in $(PhTe)_2$-injected rats has been demonstrated by immunohistochemical approaches. In addition, MAPK pathway might be a link between altered IF equilibrium and neural cell damage, since MAPK is implicated in IF hyperphosphorylation and neurodegeneration as well in the brain structures attained by $(PhTe)_2$ toxicity. Further supporting the cytoskeleton as an end point of neurotoxicity, hyperphosphorylated NFs can inhibit their proteolytic breakdown by calpain, a Ca^{2+}-activated protease. In addition, abnormally phosphorylated NFs accumulate in the perikarya and the phospho-NF aggregates can thus become cytotoxic by the enduring impairment of axonal transport of NFs (see **Figure 2**). The increased time the NF spent in the

cell body is thought to result in further aberrant phosphorylation and may prevent them from entering the axon, resulting in a deleterious feedback loop [24].

In summary, we propose that complex and integrated actions mediate the (PhTe)$_2$ toxicity directed to the cytoskeleton of neural cells. These molecular mechanisms induce spatiotemporal responses of the cells because of the different windows of susceptibility of the developmental brain. Nonetheless, the Ca^{2+}-initiated events highlight a role for this neurotoxicant as a disruptor of the cytoskeleton.

5. Cytoskeleton as a target of amino acids and their metabolites

Misregulated cytoskeletal homeostasis is among the molecular mechanisms underlying the neural cell dysfunction in brain tissue exposed to high levels of amino acids and/or their metabolites. In humans, several neurological impairments are associated with enzymatic deficiencies or defects in proteins involved in cellular metabolism of neural cells, causing accumulation of metabolic intermediates associated with neuronal damage. We discuss some aspects of the molecular mechanisms underlying the disruption of cytoskeletal homeostasis in response to branched-chain keto acids (BCKAs) derived from leucine, isoleucine and valine. We also addressed the effects of homocysteine and quinolinic acid (QUIN), a metabolite of tryptophan metabolism, directed to the cytoskeleton.

5.1. Branched chain α-keto acids and the cytoskeleton of neural cells

The branched-chain ketoacids, α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-keto-isovaleric acid (KIV) are produced from the respective branched-chain amino acids (BCAAs) leucine, isoleucine and valine, in the reaction catalyzed by the branched-chain α-keto acid dehydrogenase (BCKAD) complex. A deficiency of the BCKAD complex is an inherited metabolic disease known as maple syrup urine disease (MSUD) which lead to the accumulation of BCAAS and BCKAs in tissues and body fluids resulting in dramatic cerebral symptoms [25].

Curiously, cortical slices of young rats exposed to high levels of the BCAAs individually preserve the homeostasis of the cytoskeleton. On the other hand, their respective keto acids provide an interesting example of the fine-tune regulation of the cytoskeleton, since KIC [26] and KMV [27] were differently deleterious to the homeostasis of the cytoskeleton. KIC and KMV alter the dynamics of IF proteins of astrocytes and neurons through different transduction mechanisms dependent on excessive intracellular Ca^{2+} influx, while KIV appears not to be involved in the disruption of the IF cytoskeleton [28].

The effect of KIC is outlined by hypophosphorylation of GFAP, NF-M and NF-L in very young rats (up to 12 days of age) changing to hyperphosphorylation of the same proteins later in development (17 days of age). Nonetheless, both responses of the cytoskeletal-associated phosphorylating system are regulated by Ca^{2+} currents through the NMDA and L-VDCC, as well as by the intracellular Ca^{2+} storage release from the ER, leading to a differential activation of protein phosphatases or kinases [28]. These paradoxical findings provide an interesting

insight into the differential susceptibility of cortical IF cytoskeleton to the exposure to pathological levels of this metabolite. The different vulnerabilities of the cytoskeleton of cortical cells during development might be ascribed to the temporal maturation mediated by a multitude of developmental processes and signaling pathways. It is conceivable that they are associated with the pathological role of the developmentally regulated glutamate receptors in neural cells since the expression patterns of glutamate receptor subunit genes change during the ontogeny of the brain. Distinct regional and temporal patterns of the expression of types and subtypes of the glutamate ionotropic receptors during ontogeny may possibly explain the different signaling pathways targeting the cytoskeleton of cortical neural cells during development.

Interestingly, KMV disturbs the IF-associated cytoskeletal phosphorylation only in 12-day-old rats without changing the phosphorylation level of these proteins in younger or older animals, showing a specific window of vulnerability of cytoskeleton to KMV insult in the cerebral cortex of developing brain. Strikingly, this effect was dependent on intracellular Ca^{2+} concentrations; however, in this case ɣ-amino butyric acid A and B ($GABA_A$ and $GABA_B$, respectively) rather than glutamate receptors were involved in this action. This is in agreement with $GABA_A$ and $GABA_B$ receptors mediating the induction and maintenance of Ca^{2+} levels [27].

Overall, we propose that BCKAs in supra-physiological concentrations disrupt the cytoskeleton of rat brain through misregulation of the phosphorylating system associated with the IF cytoskeleton. We evidenced developmentally regulated mechanisms in which Ca^{2+}-mediated excitotoxicity plays a critical role in destabilizing the cytoskeleton that may ultimately disrupt normal cell function and viability. Although evidence from animal models should be taken with caution, we can propose that the disrupted cytoskeleton is part of the physiopathology of MSUD.

5.2. Hyperhomocysteinemia and the cytoskeleton of neural cells

Homocysteine (Hcy) is a sulfur-containing amino acid generated during methionine metabolism. Genetic mutations impairing Hcy metabolism cause accumulation of this amino acid attaining high levels in blood, leading to severe hyperhomocysteinemia and brain damage. Otherwise, along with genetic factors, mild-moderate hyperhomocysteinemia is associated with nutritional imbalance and hormonal factors. Mild hyperhomocysteinemia, which markedly enhance the vulnerability of neuronal cells to excitotoxicity and oxidative imbalance, is also common in older people, constituting an independent risk factor for stroke and cognitive impairment [29].

Various existing experimental evidences from our group link hyperhomocysteinemia and cytoskeletal misregulation, supporting that disrupted cytoskeleton could be an end point of neural dysfunction in this neurometabolic disorder. Experiments with brain slices acutely exposed to mild Hcy levels (100 μM) showed greater vulnerability of hippocampal cytoskeleton as compared with cortical one. Moreover, a window of vulnerability of the cytoskeleton of hippocampal cells is evidenced, since misregulated phosphorylation is detected only at postnatal day 17 [30], reflecting an altered activity of the endogenous phosphorylating system associated with the IFs in this brain structure. As expected, NMDA receptors, L-VDCC and extracellular Ca^{2+}influx result in PKC and PKCaMII activation. The prevention of Hcy action through the inhibition of PKC and MEK, a step that is upstream of MAPK cascade (Raf-1/MEK/MAPK),

is consistent with an effect at the level of the monomeric GTPase Raf-1, supporting a role for PKC phosphorylating and activating Raf-1 in the Hcy-induced modulation of the cytoskeleton.

In contrast with hypophosphorylation found in hippocampal slices, the chemically induced chronic hyperhomocysteinemia differently alters the signaling mechanisms directed to the cytoskeleton, producing PP1-, PP2A- and PP2B-mediated hypophosphorylation of NF sub-units and GFAP in hippocampal slices of 17-day-old rats without affecting the cerebral cortex [31] through glutamate and Ca^{2+}-mediated mechanisms. Further evidence that homocysteine targets the cytoskeleton came from cytoskeletal reorganization in primary astrocytes and neurons exposed to homocysteine [32]. Dramatically altered actin cytoskeleton in primary astrocytes exposed to 100 μM Hcy is consistent with the role of actin as a main determinant of cell morphology. Concomitant disrupted GFAP meshwork underlies the remodeled actin cytoskeleton and altered cell morphology. These findings provide further evidence of the cross-talk among the different cytoskeletal subsystems and the roles played by the toxic levels of Hcy.

Therefore, taking into account our experimental evidence it is conceivable that disturbed cell signaling is an important determinant of the disrupted homeostasis of the cytoskeleton as a whole, with widespread consequences on cell function that could be associated with human hyperhomocysteinemia.

6. Cytoskeleton is a target of quinolinic acid neurotoxicity

Quinolinic acid is a neuroactive metabolite of the kynurenine pathway normally found in nanomolar concentrations in human brain and cerebrospinal fluid (CSF). QUIN is antagonist of NMDA receptor and it has a high *in vivo* potency as an excitotoxin supporting involvement in the pathogenesis of a variety of human neurological diseases. The neurotoxicity of QUIN results from complex mechanisms including presynaptic receptors, energetic dysfunction, oxidative stress, transcription factors and behavior [33]. We experimentally demonstrate that the disruption of the cytoskeleton, in particular, misregulation of the phosphorylation system associated with the IFs, is a target of QUIN toxicity in injected rat striatum, tissue slices and primary astrocytes and neurons in culture.

6.1. Effects of intrastriatally injected quinolinic acid on the cytoskeleton of neural cells

Acute intrastriatal injection of QUIN (150 nmol/0.5 μL) in adolescent rats (30 days old) provokes NF-L and GFAP hyperphosphorylation 30 min after infusion, evidencing the susceptibility of the cytoskeleton of both neurons and astrocytes in the early events of QUIN toxicity. Hyperphosphorylated NF-LSer55 destabilizes the NF structure and this might represent an early step in the pathophysiological cascade of deleterious events exerted by QA in rat striatum. Experimental insights to get light on the molecular mechanisms underlying this effect point to NMDA-mediated Ca^{2+} events and oxidative stress upstream of activated second messenger-dependent protein kinases PKA, PKC and PKCaMII, but not MAPKs after QUIN infusion [34].

A link between misregulation of cell-signaling mechanisms, disruption of IF phosphorylation and cell damage as part of QUIN toxicity becomes more evident analyzing the

long-lasting effect of the acute intrastriatal injection of QUIN in adolescent rats on the dynamics of the phosphorylating system until 21 days after injection [35]. The acutely injected QUIN alters the homeostasis of IF phosphorylation in a selective manner, progressing from striatum to cerebral cortex and hippocampus. Twenty-four hours after QUIN injection, the IFs are hyperphosphorylated in the striatum. This effect progresses to cerebral cortex causing hypophosphorylation at day 14 and appears in the hippocampus as hyperphosphorylation at day 21 after QUIN infusion, PKA and PKCaMII mediating this effect. However, MAPKs (Erk1/2, JNK and p38MAPK) are hyperphosphorylated/activated only in the hippocampus, suggesting different signaling mechanisms in these two brain structures during the first weeks after QUIN infusion. Also, PP1 and PP2B-mediated hypophosphorylation of the IF proteins in the cerebral cortex 14 days after QUIN injection reinforces the selective signaling mechanisms in different brain structures. Increased GFAP immunocontent in the striatum and cerebral cortex 24 h and 14 days after QUIN injection, respectively, suggests reactive astrocytes in these brain regions. Yet, we observe biochemical and histopathological alterations in the striatum, cortex and hippocampus, as well as altered behavioral tests in response to the long-lasting exposure to QUIN through glutamate and Ca^{2+}-mediated mechanisms. Thus, it is tempting to propose that the long-lasting deleterious effect of intrastriatal QUIN injection could be due to the fact that QUIN interferes with the highly regulated signaling mechanisms targeting the cytoskeleton in the immature brain [36].

6.2. Insight into the molecular basis of quinolinic acid action toward the cytoskeleton

Studies in acute brain slices further support the role of glutamatergic signaling and Ca^{2+} overload disturbing the cytoskeletal equilibrium downstream of QUIN exposure. Moreover, this experimental approach brings light on the cell-specific mechanisms targeting the cytoskeleton in astrocytes and neurons when the cell connections are partially preserved. In astrocytes, the QUIN action is mainly due to increased Ca^{2+} influx through NMDA and L-VDCC. In neuronal cells, QUIN acts through the activation of metabotropic glutamate receptors and influx of Ca^{2+} through NMDA receptors and L-VDCC, as well as Ca^{2+} release from intracellular stores. These mechanisms then set off a cascade of events including the activation of PKA, PKCaMII and PKC, which phosphorylate head domain sites on GFAP and NFL. Moreover, Cdk5 is activated downstream of mGluR5, phosphorylating the KSP repeats on NFM and NFH. Metabotropic glutamate receptors type 1 (mGluR1) is upstream of PLC, which, in turn, produce DAG and IP3 promoting hyperphosphorylation of KSP repeats on the tail domain of NFM and NFH [37].

6.3. The cytoskeleton of astrocytes and neurons responds differently to quinolinic acid toxicity

The susceptibility of the cytoskeleton to toxic levels of QUIN is also detectable in isolated astrocytes and neurons growth in primary cultures [38]. In astrocytes, Ca^{2+}-mediated glutamate mechanisms target the endogenous phosphorylating system, since metabotropic glutamate receptors and Ca^{2+} influx through NMDA receptors are upstream of PKA, PKCaMII and PKC activation, provoking GFAP hyperphosphorylation. Interestingly, the misregulated phosphorylation system leads to a reversible and dramatically altered actin cytoskeleton with concomitant change

of morphology to fusiform and/or flattened cells with retracted cytoplasm and disruption of the GFAP meshwork [39] supporting the dynamic behavior of the cytoskeleton.

Interestingly, neurons show greater vulnerability to QUIN than astrocytes (10×). Neurons exposed to QUIN presented PKA- and PKC-mediated hyperphosphorylation of NF subunits. These effects are also downstream of ionotropic and metabotropic glutamate signaling and Ca^{2+} influx through NMDA receptors and L-VDCC. The misregulated signaling pathways disrupt the neuronal cytoskeleton, evaluated by altered neurite/neuron ratios and neurite outgrowth. It is important to consider that microtubules play a central role in cell polarity [40]. In particular, microtubules are the main determinants of neuronal polarity and regulation of microtubule dynamics includes tubulin posttranslational modifications [40] and phosphorylation of microtubule-associated proteins (MAPs), whose binding to microtubules is essential for neurite formation [41]. As an example, activated GSK-3β leads to increased phosphorylation of some MAPs, destabilizing microtubules with consequence for neurite stabilization [42]. Therefore, the neurite destabilization could derive from both NFs and microtubules disruption.

Interestingly, we found a protective role of astrocyte-conditioned medium on the disrupted neuronal cytoskeleton and morphometric alterations, suggesting that QUIN-induced trophic factors secreted by astrocytes are able to modulate signaling mechanisms targeting the neuronal cytoskeleton. More interestingly, co-cultured astrocytes and neurons preserve their cytoskeletal organization and cell morphology together with unaltered activity of the phosphorylating system associated with the cytoskeleton. In other words, co-cultured astrocytes and neurons tightly and actively interact with one another reciprocally protecting themselves against QUIN injury [38]. This evidence raise the question about the role played by the activated microglia eliciting signals essential to destabilize the astrocytic and neuronal cytoskeleton but this hypothesis remains to be clarified.

All together, we conclude that among the multiple mechanisms through which accumulated QUIN is able to induce cell damage, our experimental evidence points to Ca^{2+}-mediated mechanisms directed to the cytoskeletal disruption as an end point of QUIN toxicity. Both *in vivo* and *ex vivo* approaches clearly demonstrate a wide spectrum of misregulated signaling mechanisms downstream of QUIN action directly affecting the cytoskeleton and disrupting cell homeostasis. We also provide evidence that impaired physiological equilibrium of the signaling cascades directed to the cytoskeleton underlies QUIN cytotoxicity and is associated with neurodegeneration. The *in vitro* results showing disorganized cytoskeleton and altered cell morphology further support the cytoskeleton as a hallmark of stress condition that could be implicated in the human brain disorders associated with high QUIN levels.

7. Cytoskeleton of neural cells is a target of thyroid hormones

Thyroid hormones are essential for the development and function of central nervous system. In brain, these hormones are essential for myelination [43, 44], neuritogenesis [45], synaptic plasticity [46–48], IF phosphorylation [49–54], cell differentiation and maturation [55]. Considering the role of these hormones on brain development, thyroid diseases might account for brain injury as well as alteration in mood and cognition [56].

The classical mechanism of thyroid hormone action involves the modulation of nuclear receptors by 3,5,3′-triiodo-L-thyronine (T_3). The nuclear receptors are ligand-dependent transcription factors, which are involved in the genomic-dependent effects of thyroid hormones. However, there are numerous physiological effects of these hormones that cannot be mediated by the genome-like mechanism, due the short time frame in which the response occurs. Nongenomic actions of thyroid hormones are defined as events that (i) do not primarily involve the cell nucleus, (ii) are rapid in onset (minutes or a few hours) relative to transcription and translation and (iii) do not require gene transcription and protein synthesis [57]). These events are triggered by rapid/nongenomic responses that are frequently associated with secondary messenger-signaling pathways.

7.1. Insight into the molecular basis of genomic and nongenomic action of thyroid hormones toward the cytoskeleton of neural cells

The first evidence of nongenomic actions of thyroid hormones targeting the cytoskeleton demonstrated the thyroxine-dependent modulation of actin polymerization in cultured astrocytes. Thyroxine (T_4) was involved in the conversion of soluble actin to a fibrous form through nongenomic mechanism [58].

While many of the T_3 actions are mediated by genomic-dependent mechanisms, T_4 and reverse 3,3′,5′-triiodothyronine (reverse T_3, rT_3) exert direct, The nongenomic effects in neural cells. Both T_4 and rT_3 hormones control actin polymerization in cultured astrocytes without affecting gene expression. The authors suggested that these events might contribute to thyroid hormone's influence on brain development. Subsequently, the same research group showed that both T_4 and rT_3, but not T_3, directly regulate the F-actin content of elongating neurites of cerebellar neurons. These results provide a molecular mechanism for the influence of thyroid hormones on brain development that is independent of regulated gene expression [59].

Trentin and Moura Neto [60] demonstrated that T_3 altered the organization of GFAP in cerebellar astrocytes in culture. GFAP filaments that normally spread in the cytoplasm of astrocytes became organized around the cell nucleus. In addition, Zamoner and coworkers [51] showed that both T_3 and T_4 induced GFAP phosphorylation and reorganization in glioma C6 cells through the inhibition of RhoA GTPase. The modulation of GFAP was accompanied by increased proliferation of glioma cells. Taking together, these results suggest that thyroid hormones may be important regulators of astrocyte growth and differentiation.

Despite the evidence that nongenomic actions of thyroid hormones initiated at the plasma membrane via integrin $\alpha V\beta3$ [57, 61], the complexity of the processes underlying the differential mechanisms of action to thyroid hormones suggests the existence of multiple binding sites for these hormones. In this context, it has been previously demonstrated that both T_3 and T_4 may modulate the GABAergic system and induce PKA- and PKCaMII-mediated hyperphosphorylation of vimentin, GFAP, NF-M and NF-L in cerebral cortex from very young rats (up to 10 days of age) [50]. However, only T_4 caused hyperphosphorylation of the same proteins later in development (15 days of age) through GABA-independent mechanisms [49]. These paradoxical findings provide an interesting insight into the differential susceptibility of cortical IF cytoskeleton to thyroid hormone exposure.

Calcium-dependent mechanisms play a central role on the thyroid hormone-induced modulation of the phosphorylating system associated with IFs. Zamoner and colleagues [49] demonstrated that the nongenomic mechanisms underlying the effects T_4 targeting the IF-associated phosphorylating system in cerebral cortex from 15-day-old rats are dependent on extracellular Ca^{2+} influx through VDCC, as well as Ca^{2+} release from ER stores.

Taking into account that in rat the myelination peak is coincident with postnatal day 15 and that this is a period of intense synaptogenesis, the NF hyperphosphorylation induced by T_4 in cerebral cortex from 15-day-old rats appears to be correlated to synaptogenesis and myelination (for review, see [53]).

In summary, we could suggest that nongenomic actions of T_4 targeting the cytoskeleton of glial cells and neurons might account for neuronal cell migration, myelination, synaptogenesis and synaptic plasticity. Moreover, the modulation of NF phosphorylation by thyroid hormone may control axonal caliber.

7.2. Hypothyroidism and the cytoskeleton of neural cell

The effects of thyroid hormones in central nervous system during development include the modulation of the cytoskeleton dynamics. Hypothyroidism in the developing rat brain is associated with oxidative stress and aberrant intraneuronal accumulation of NFs in the perikaryon of Purkinje neurons (see **Figure 2**). The authors suggested that the neuron alterations observed in the developing hypothyroid brain are comparable to those seen in neurodegenerative diseases [62]. Corroborating these findings, it has been shown that the effects of hypothyroidism on neuronal cytoskeleton involve the developmental modulation of specific isoforms of protein expression, which induce stoichiometric imbalance between the NF triplet [52]. In addition, thyroid hormone deficiency induces a delay and a partial arrest of astrocyte differentiation, supported by the decreased expression of GFAP both in cortical [52] and in hippocampal astrocytes [54], which was accompanied by downregulation of the astrocyte glutamate transporters. These findings are associated with the extracellular signal-regulated kinase (ERK)1/2 and c-jun terminal kinase (JNK) activation. NF hyperphosphorylation might account for the aberrant intraneuronal accumulation of these cytoskeletal structures previously described [62].

Our research group demonstrated the hyperphosphorylation of tail KSP repeats on NF-H in hypothyroid cortical and hippocampal neurons [52, 54]. The carboxyl-terminal phosphorylation of NF-H progressively restricts association of NFs with kinesin, the axonal anterograde motor protein and stimulates its interaction with dynein, the axonal retrograde motor protein [63]. This event could represent one of the mechanisms by which carboxyl-terminal phosphorylation would slow NF axonal transport.

Taking into account our experimental evidence, we propose that the consequences of congenital hypothyroidism to neural cells involve IF hyperphosphorylation, misregulation of glutamate-glutamine cycle, oxidative stress and glutamate excitotoxicity. These events suggest a compromised astroglial defense system that is probably playing a role in the physiopathology of the neurological dysfunction of hypothyroidism (**Figure 3**).
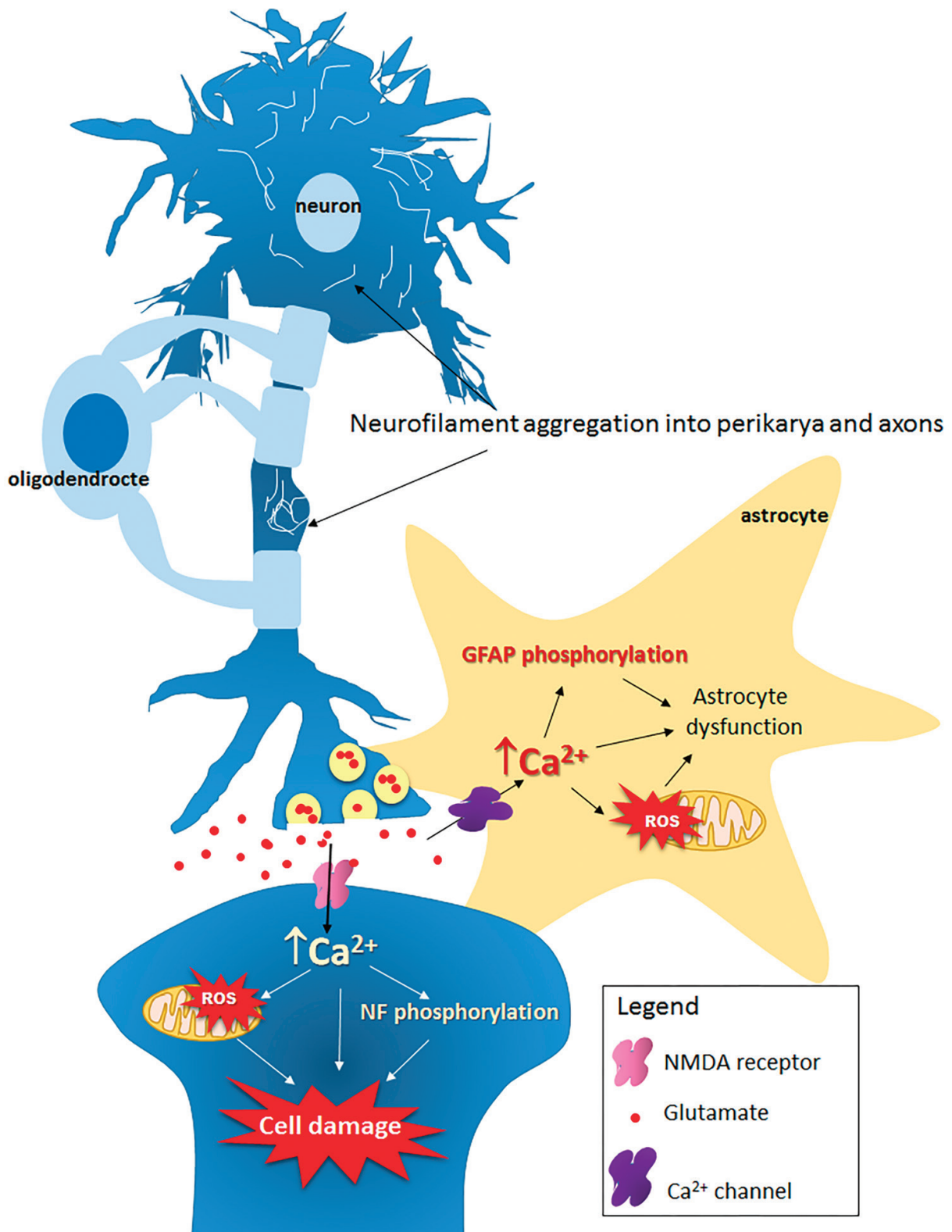
Figure 3. Role of glutamate excitotoxicity on intermediate-filament dynamics and cell damage. Congenital hypothyroidism leads to glutamate excitotoxicity, calcium overload, and oxidative stress. These events are related to intermediate-filament (GFAP and NF) hyperphosphorylation and neural cell damage.

8. General conclusion

Studies of our group on the endogenous phosphorylating system associated with the IF proteins of neural cells point to a critical role of disrupted cytoskeleton in response to a variety of signals both in physiological and in pathological conditions. Our findings highlight the IFs as a preferential target of the signal transduction pathways. Importantly, a large body of evidence shows a link among misregulation of cell-signaling mechanisms, disruption of IF phosphorylation and cell damage in response to different stress signals. While the exact signaling pathways regulating NF phosphorylation remains elusive, there is increasing evidence that known signal transduction cascades are involved. These actions can be initiated by the activation of NMDA-, L-VDCC, or G protein-coupled receptors and the signal is transduced downstream of Ca^{2+} mobilization or monomeric GTPase activation through different kinase/phosphatase pathways, regulating the dynamics of the cytoskeleton. **Figure 4** summarizes
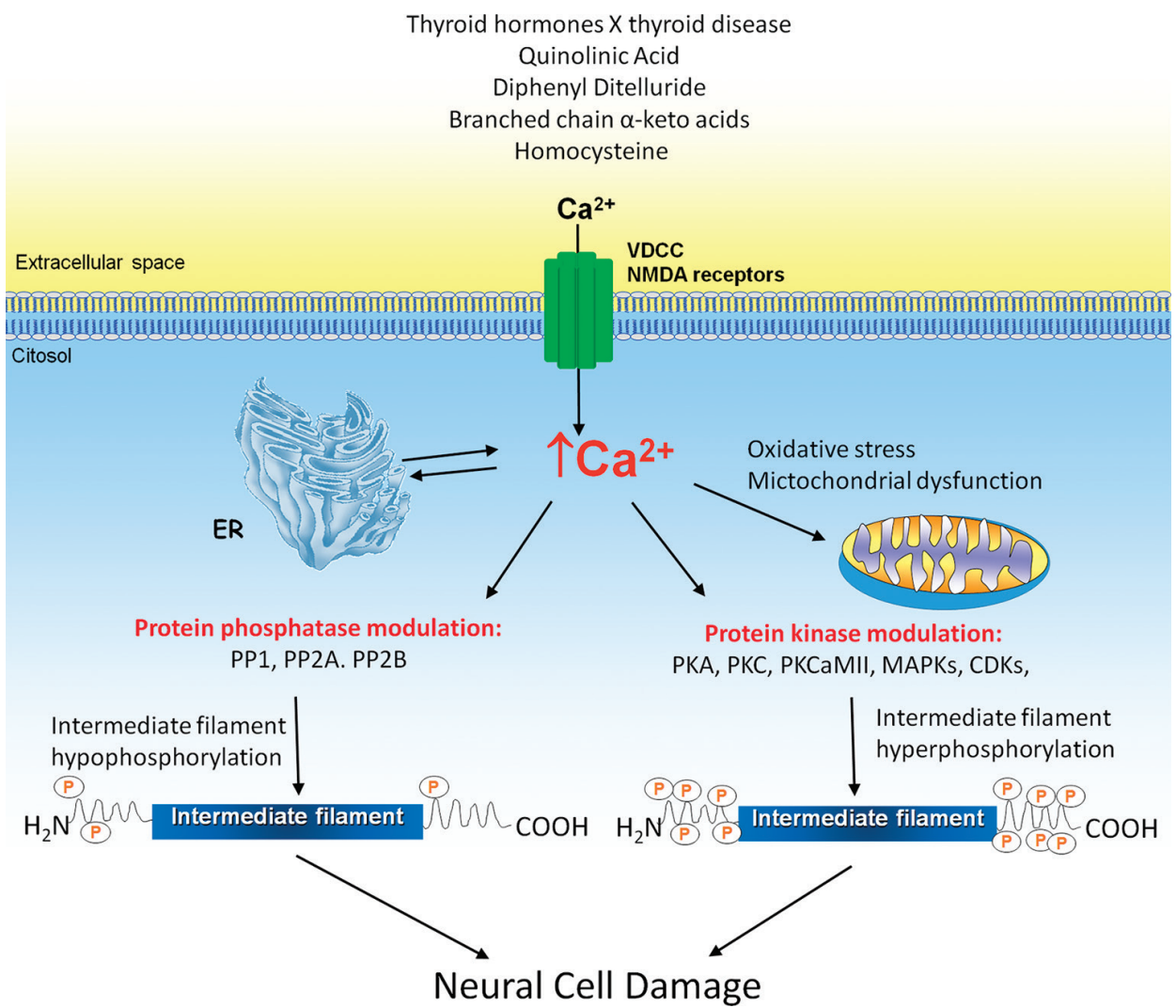

Figure 4. Summary of calcium-associated mechanisms triggered by thyroid hormones, quinolinic acid, diphenyl ditelluride, branched-chain keto acids, and homocysteine targeting intermediate-filament phosphorylation in neural cells. Calcium influx through the NMDA receptor or voltage-dependent calcium channels (VDCC) can be responsible for the activation of lethal metabolic pathways in neural cells. Augmented intracellular Ca^{2+} levels might be associated with the modulation of diverse cell-signaling pathways and exhibit a diverse range of responses to their stimuli.

the calcium-associated mechanisms triggered by thyroid hormones, quinolinic acid, $(PhTe)_2$, BCKAs and homocysteine targeting IF phosphorylation in neural cells.

Despite the focus on the misregulation of IF dynamics in response to signaling mechanisms downstream of metabolites and neurotoxicants, we should consider that cytoskeleton is a complex meshwork of interconnecting filaments [1]. In this regard, the morphological alterations demonstrated in primary cells in culture mainly reflect the reorganization of the meshwork of filaments. Taking into account our findings, we propose that misregulation of kinase/ phosphatase cascades downstream of stressors could disrupt the cytoskeleton as a whole and this might be an important determinant of neural dysfunction associated with the action of neurotoxicants and in neurometabolic conditions.

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