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Mini Review

Calcium transporters and their role in the development of neuronal disease and neuronal damage

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Abstract. Neurodegeneration comprises assembly of pathophysiological events that gives rise to a progressive loss of neuronal structure and function including cellular damage, diseases development or cellular death. Neurons respond by adjusting signaling pathways, from gene expression to morphological changes. In most of these processes, Ca^{2+} signaling plays a pivotal role. By increasing the Ca^{2+} concentration, the cell responds to neuronal, neurotrophic and other growth factor stimuli, however, the molecular mechanism of Ca^{2+}-dependent neurite outgrowth and development yet requires further elucidation.

Here we focus on the role of Ca^{2+} and selected Ca^{2+} transporters involved in processes of CNS neurodegeneration – inositol 1,4,5-trisphosphate (IP_3Rs) and ryanodine receptors (RyRs), considering the fact that these receptors may be important "sensors" of disturbed intracellular calcium homeostasis. We propose that *in vitro* cellular models could serve as suitable experimental systems for the determination of the role that these receptors play in neuropathological conditions.

Recognition of the principles, key players and regulatory processes may elucidate the role of Ca^{2+} in the regulation of neuronal proliferation, development and differentiation, growth and axon navigation in neurodegenerative and regenerative processes. This may provide a new insight and also discovery of novel therapeutic-targeting possibilities for severe neurological disorders and pathophysiological changes.

Key words: Neuronal diseases — Neuronal injury — Neurodegeneration — Intracellular Ca^{2+} transporters — *In vitro* cellular model

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; CICR, calcium-induced calcium release; ER, endoplasmic reticulum; IP_3Rs, inositol 1,4,5-trisphosphate; MSNs, medium spiny striatal neurons; PS, presenilin; ROS, reactive oxygen species; RyRs, ryanodine receptors; SCAs, spinocerebellar ataxias.

Calcium signaling and homeostasis within neurons

Calcium is a second messenger that facilitates a variety of neuronal cellular processes from gene expression (West et al. 2001), neurotransmitter release (Catterall and Few 2008) and synaptogenesis (Michaelsen and Lohmann 2010) to apoptosis (Paschen 2003). The regulation of Ca^{2+} homeostasis is critical for normal cellular function and survival. Prolonged disruption of cytoplasmic Ca^{2+} concentration is usually detrimental (Verkhratsky and Toescu 2003).

Under physiological conditions, the activation of presynaptic neurons leads to the release of neurotransmitters to the synaptic cleft *via* Ca^{2+}-dependent process. Released neurotransmitters activate receptors in the membrane of the subsequent neurons and initiate signal transmission. Ca^{2+} fluxes across the plasma membrane and between the intracellular compartments play a critical role in the fundamental functions of neurons. In response to stimuli including membrane depolarization, mechanical stretching, noxious insults, extracellular agonists, intracellular messengers or depletion of intracellular Ca^{2+} stores, Ca^{2+} signals are generated which

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regulate neurite outgrowth, growth cone guidance (Gomez and Spitzer 1999), synaptogenesis, synaptic transmission, plasticity and cell survival (Kawamoto et al. 2012).

The cytoplasmic concentration of Ca^{2+} increases *via* transient signals, which can range from 50–300 nM at rest to 1–500 mM upon activation (Kawamoto et al. 2012). Whereas the extracellular Ca^{2+} concentration is around 1.2 mM, intracellular concentrations are approximately 10 000-folds lower and reach a concentration of about 100 nM. Membrane depolarization leads to a significant increase in intracellular Ca^{2+} levels and affects the membrane potential. Ca^{2+} is a key element that can relay information from the change in plasma membrane potential to the biochemical and metabolic response of the cell.

In the plasma membrane, there are two principal paths of Ca^{2+}entry into the cell: ionotropic receptor-operated (ligand-gated) channels (ROCs), and voltage-operated Ca^{2+} channels (VOCCs). These channels play important and active roles in the integration of electrical signals and affect the rate and timing of action potentials and the shape and firing patterns (Huguenard 1996). The low-voltage activated channels also facilitate long-term changes in neuronal plasticity (Ikeda et al. 2003) and regulate various intracellular molecules including protein kinase A and C, protein tyrosine kinase (PTK) and calcium/calmodulin-dependent protein kinase II (CaMKII), which is involved in the regulation of transcription (Wolfe et al. 2002).

There are four known representatives of L-type calcium channels (LTCC): $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ and $Ca_V1.4$. Their two subtypes $Ca_V1.2$ and $Ca_V1.3$ have an unique role in neuronal activity. $Ca_V1.2$ channels in neurons have an activation threshold around –30 mV (Bourinet et al. 1994). $Ca_V1.3$ channels are activated at more negative voltages around –40 mV (Koschak et al. 2001), have longer inactivation and are less sensitive to dihydropyridine antagonists compare to $Ca_V1.2$ channels. The LTCC controls the expression of CREB (cAMP response element-binding protein), which is responsible for key functions of the CNS, such as memory, learning and critical signal integration (Deisseroth et al. 2003; Moosmang et al. 2005; Lacinova at al. 2008).

The endoplasmic reticulum (ER) and mitochondria are the main intracellular calcium stores that contribute to the influx of intracellular calcium. Calcium released from the ER amplifies or triggers calcium rises that are themselves initiated by influx from the plasmalemma. Mitochondria are organelles that supply energy to cells in the form of adenosine triphosphate (ATP) and contribute to the regulation of calcium homeostasis, fatty acid oxidation, steroid synthesis and apoptosis (Turner and Schapira 2010). They play a prominent role in determining the shape, amplitude and duration of the transient rise in intracellular Ca^{2+} (Kawamoto et al. 2012). Increasing evidence suggests that mitochondrial dysfunction plays an important role in brain ageing and the pathogenesis of neurodegenerative diseases (Eckert et al. 2012). Dysfunction of single enzyme complexes, production of reactive oxygen species (ROS), mitochondrial permeability transition pore opening (mPTP), enhanced apoptosis, and structural alterations of mitochondria are believed to be crucial for the onset and progression of neurodegenerative diseases (Bilsland et al. 2008).

The contribution of Ca^{2+} to cytosol from intracellular stores is mediated *via* two types of receptors: inositol-1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) (Verkhratsky 2005), which are ubiquitously expressed. Calcium exposure at these channels can facilitate subsequent release by calcium-induced calcium release (CICR). Given the complexity of interactions among calcium sources and their degenerative/regenerative potential, neurons regulate calcium signaling tightly to ensure proper function and viability (Goussakov et al. 2010).

Ca^{2+} release through IP_3Rs requires binding of the second messenger IP_3, which is generated by phospholipase C (PLC) in response to the activation of various G-protein-coupled receptors (GPCRs) or tyrosine kinase-linked receptors in the cell membrane. IP_3Rs constitute a family of three paralogs (IP_3R1, IP_3R2, IP_3R3), and they mediate cell physiological processes ranging from gene transcription to forming learning traces for memory (Foskett et al. 2007). In neurons, type 1 IP_3R is expressed predominantly (Furuichi et al. 1993) and its gene expression is regulated by synaptic activity. Mice lacking IP_3R1 display severe ataxic behavior (Matsumoto et al. 1996), and mice with a spontaneous mutation in the IP_3R1 gene experience convulsions and ataxia, suggesting a major role of IP_3R1 in neuronal function. In neuronal HT22 cells, IP_3R2 was found predominantly in the nuclear envelope and IP_3R1 and IP_3R3 in the ER (Duncan et al. 2007).

Ryanodine receptors, similarly as IP_3Rs, are also found in three paralogs (RyR1, RyR2, and RyR3). They transport Ca^{2+} into the cytosol by binding Ca^{2+} on its cytosolic side thus establishing a positive feedback mechanism. A small amount of Ca^{2+} in the cytosol near the receptor triggers RyR to release even more Ca^{2+} (CICR).

In neurons, RyRs are especially important. The localized and time-limited activity of Ca^{2+} in the cytosol is also called a Ca^{2+} wave. The wave is built *via* the feedback mechanism of GPCR- or TRK-mediated RyR activation of PLC, which leads to the production of IP_3 that in turn activates the IP_3 receptor.

The distribution of IP_3Rs in pyramidal neurons is predominantly in the soma and proximal dendrites, whereas RyRs are also found in distal processes and spines (Fitzpatrick et al. 2009).

Ca^{2+} reuptake and balance is regulated by SERCA pumps (sarco-endoplasmic reticulum Ca^{2+} ATPase), which maintain a steady state Ca^{2+} concentration within the lumen of the ER (Kawamoto et al. 2012).

Neurodegeneration

Pathology of central nervous system in adults is typically associated with a limited ability of the axon to regenerate. Currently, there is a general agreement that mitochondrial dysfunction, oxidative stress, neuroinflammation, impaired protein degradation, axonal transport and apoptosis are involved in neurodegeneration. Neuronal injury, lesions, infection and neuronal disease development involve cascades of pathophysiological processes including modulation of cellular signaling systems that typically result in permanent damage and neurodegeneration. Serious diseases including Alzheimer's disease, Huntington's disease, spinocerebellar ataxias (SCAs) and dementia, amyotrophic lateral sclerosis or prion diseases may develop. The pathology of these diseases is associated with selective neuronal vulnerability and degeneration.

In each disease and disorder, neurons gradually lose function as the disease progresses over time, and it is associated with lost function of regulatory mechanisms culminating in mitochondrial DNA mutations and oxidative stress (Lin and Beal 2006). Many of these diseases are late-onset and aging is one of the greatest risk factors for their development. Dysregulation of cytoplasmic and mitochondrial Ca^{2+} homeostasis has emerged as a common molecular mechanism of these neurodegenerative disorders despite their different etiologies (Bezprozvanny 2009).

Calcium dysregulation in neuronal diseases

Dysregulated calcium signaling is important for the pathogenesis of Alzheimer's disease, Huntington's disease, spinocerebellar ataxias and other diseases through a variety of mechanisms (LaFerla 2002; Stutzmann et al. 2006; Bezprozvanny and Mattson 2008). The most pronounced effects are associated with mutant proteins that participate in the regulation of ER calcium release.

Alzheimer's disease is a common form of dementia involving slowly developing and ultimately fatal neurodegeneration. Besides age, which is the main risk factor, accumulation of extracellular β-amyloid plaques, intracellular neurofibrillary tangles, and neuronal loss, mutations in presenilins (PS) and the amyloid precursor protein and disrupted intracellular Ca^{2+} homeostasis have been implicated. The manifestation of Ca^{2+} dysregulation is presented as an attenuated capacitive Ca^{2+} entry (Herms et al. 2003) but typically due to exaggerated Ca^{2+} release from the ER (Stutzmann et al. 2007). The mechanisms of exaggerated ER Ca^{2+} release have been ascribed to enhanced loading of the ER lumen due to disruption of the putative wild-type PS Ca^{2+} channel function (Nelson et al. 2007) or to enhanced activity of the SERCA (Green et al. 2008). Enhanced release from stores has been attributed either to enhanced expression of Ca^{2+} release channels (Chakroborty et al. 2009) or, in the case of the IP_3Rs, to enhanced activity in response to its ligand IP_3 (Cheung et al. 2010).

In familial Alzheimer's disease, mutant PS1 and PS2 have been described (Cheung et al. 2008). Single channel recordings of IP_3Rs have shown that expression of mutant PS1 and PS2 is associated with an apparent sensitization of the IP_3R channel to IP_3, resulting in enhanced channel gating (Cheung et al. 2008), which suggests that modulation of IP_3R channel gating is a fundamental mechanism that exaggerates Ca^{2+} signaling in familial AD PS-expressing cells. It is expected that enhanced IP_3R channel gating and the resulting exaggerated Ca^{2+} signaling caused by biochemical and functional interactions of mutant PS and IP_3Rs is disease-specific and a predominant mechanism in Alzheimer's disease. In addition, exaggerated Ca^{2+} signaling through this mechanism results in enhanced generation of ROS, believed to be another important component in Alzheimer's disease pathogenesis (Reddy and Beal 2005).

Several recent studies have also shown that RyR-evoked calcium release is preferentially enhanced by PS mutations *via* increased RyR2 expression (Stutzmann et al. 2006; Chakroborty et al. 2009). In contrast, channel studies have shown that mutant PS alters IP_3R gating properties (Cheung et al. 2008, 2010) or causes ER channel leakage (Nelson et al. 2007). Regardless of the mechanism, the functional implications of increased CICR results in aberrant RyR-calcium store release. During basal synaptic transmission, this anomaly enhances pre-synaptic vesicle release and long-term plasticity in neurons (Chakroborty et al. 2009).

In Huntington's disease and spinocerebellar ataxias, a crucial role of IP_3R1 in the development of neurodegeneration has also been described. Huntington's disease and SCAs are autosomal dominant neurodegenerative disorders. Huntington's disease is caused by an expansion in polyglutamine in the amino-terminal region of the huntingtin (Htt) protein and primarily affects medium spiny striatal neurons (MSNs). SCAs are typically caused by polyQ-expansion in ataxin proteins and primarily affect cerebellar Purkinje cells. Spinocerebellar ataxias constitute a heterogeneous group of more than 30 autosomal dominant genetic and neurodegenerative disorders that are generally characterized by progressive ataxia and cerebellar atrophy. The underlying causes of neuronal dysfunction and death in Huntington's disease and the SCAs remain poorly understood. Recently, emerging results support the concept that excessive activity of IP_3Rs and abnormal Ca^{2+} signaling plays a major role in the pathogenesis of Huntington's disease and the SCAs. In parallel, it has been discovered that mutant huntingtin, ataxin-2 and ataxin-3 proteins are specifically bound to the carboxy-terminal region of the IP_3R1. It was

found that the association of mutant huntingtin or ataxins with IP_3R1 caused sensitization of the IP_3R1 to activation by IP_3 in planar lipid bilayers and in neuronal cells. Emerging research suggests that a fundamental cellular signaling pathway is disrupted by a majority of these mutant ataxin proteins, which could explain the typical death of Purkinje cells, cerebellar atrophy, and ataxia that occurs in many SCAs. Results suggest that deranged neuronal Ca^{2+} signaling might play an important role also in the pathogenesis of Huntington's disease.

The Bezprozvanny group has been heavily involved in elucidating the interactions between mutant huntingtin (mHtt) and IP_3 and IP_3R1 and other Ca^{2+}-related mechanisms relevant to Huntington´s disease (Bezprozvanny and Hayden 2004; Bezprozvanny 2009). They discovered that mHtt binds directly and specifically to the C-terminal region of IP_3R1 (Tang et al. 2003). Unbiased high-throughput screening assays confirmed that mHtt binds to IP_3R1 (Kaltenbach et al. 2007).

Interestingly, specific blockade of IP_3R1 with 2-aminoethoxydiphenyl borate (2-APB) and enoxaparin showed a neuroprotective effect (Tang et al. 2005), and disturbed Ca^{2+} signaling enhances glutamate-induced apoptosis (Zhang et al. 2008). It was demonstrated that viral delivery of a peptide that disrupts mHtt association with IP_3R1 protects MSNs *in vitro* and *in vivo* (Tang et al. 2009). Augmented IP_3R1 activity further implicates mGluR5 receptor signing in Huntington's disease pathology. Inhibitors of IP_3R1 may impede intracellular Ca^{2+} overload early in the disease state and protect MSNs from glutamate-induced excitotoxicity.

In Huntington's disease postmortem brains, alterations in ER enzymes have been observed (Cross et al. 1985). These alterations are consistent with a malfunction of ER Ca^{2+} handling in Huntington's disease MSNs. Misregulation of IP_3R1 gating and ER stress are directly implicated in Huntington's disease pathogenesis (Bauer et al. 2011).

In SCA pathophysiology, it has been proposed that the mutated proteins cause disruptions in multiple cellular pathways but the characteristic SCA pathogenesis does not begin until calcium signaling pathways are disrupted in cerebellar Purkinje cells either as a result of an excitotoxic increase or a compensatory suppression of calcium signaling (Bezprozvanny 2011).

Neurite outgrowth and dysregulated calcium signaling in neuronal damage or injury

Neurodegeneration – a common feature of serious neurological disorders – represents the loss of physiological neuronal function including correct axon and dendrite growth, synaptic plasticity and signal transduction. To understand the regulatory role of Ca^{2+} in neurological disease and development of disorders it is fundamental to understand the principles of Ca^{2+} signaling of neurite outgrowth, axon formation, individual neuron extension and formation of synapses.

A large body of evidence indicates that cytosolic Ca^{2+} has important and diverse roles in the control of axonal and dendritic growth, guidance and extension of terminal growth cones in developing neurons. Neurons maintain a baseline intracellular Ca^{2+} concentration at a resting state. Fluctuations above the resting level mediated by Ca^{2+} signaling are often linked to changes in morphology and motility. An effect on growth cone behavior depends on the spatial and temporal characteristics of the Ca^{2+} changes. Ca^{2+} effectors are stimulated by elevation in Ca^{2+} concentration and particular membrane channel types may be involved in generating Ca^{2+}changes.

During normal physiological development and differentiation, neurons break symmetry of their compartments, dendrites and axons (Barnes and Polleux 2009). Ca^{2+} signals may induce varying morphological effects on, and often oppose growth cone motility. An elevation in Ca^{2+}concentration is associated with reduced motility. Local Ca^{2+} signals promote growth cone turning (Henle et al. 2011) as they are involved in the organization and movement of actin filaments.

During axon formation, phosphatidylinositol 3,4,5-trisphosphate (PIP_3) regulation plays a critical role. Neutrophins, such as brain-derived neutrophic factor (BDNF) and neutrophin-3 (NT-3), affect tropomyosin receptor kinases, which in turn activates the downstream effectors PLC and phosphatidylinositide 3-kinase (PI3K). They produce IP_3 from phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_3 concentrates at the tip of the nascent axon. Inhibition of PI3K activity prevents axon formation in cultured neurons. Thus, PIP_3 appears to play a critical role in axon design. PIP_3 can also activate guanosinetrisphosphatase (GTPase) Rac1 and promote axon-specific actin filament remodeling (Tahirovic et al. 2010).

Arie and his group (2009) examined the mechanism underlying neurite outgrowth and its Ca^{2+} regulation during development in chick and mouse dorsal root ganglion (DRG) neurons. They found that both Ca^{2+} release from internal stores *via* RyR and Ca^{2+} influx *via* VOCCs played an important role in neurite outgrowth in chick neurons, especially at later stages of embryonic development. Thus, changes in the source of Ca^{2+} release in response to IP_3 are required for neurite outgrowth in chick neurons during development. Localized Ca^{2+} signaling in the central domain of the growth cone may be important for neurite outgrowth at early stages, and at later stages, the important Ca^{2+} signaling in neurite outgrowth may switch to CICR *via* RyR in other subregions of the growth cone, such as fillopodia and the leading edge

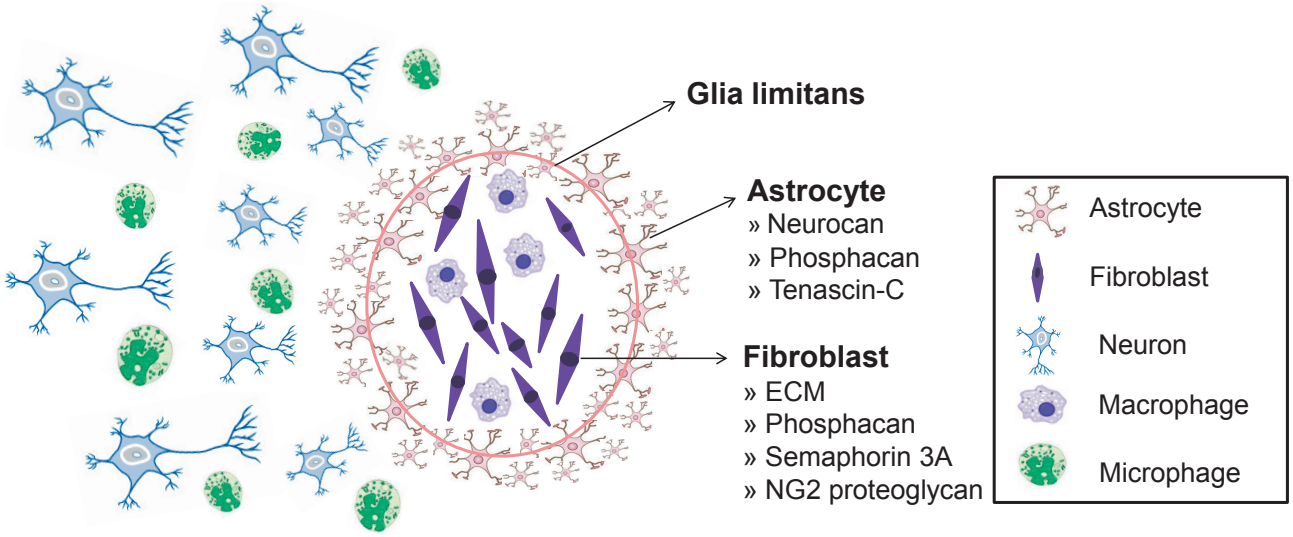

Figure 1. Schematic illustration of the cellular and molecular composition of a fibrotic scar that resembles an actual CNS lesion site. The lesion site comprises a fibrotic scar and glia limitans. Fibroblasts in the core produce extracellular matrix (ECM) including axonal growth-inhibiting molecules – phosphacan, semaphorin 3A, NG2 proteoglycan, whereas surrounding astrocytes produce neurocan, phosphacan, and tenascin-C. An *in vitro* model of a fibrotic scar mimics this composition and enables molecular and biochemical studies of underlying causes and consequences of the pathophysiology of neurodegenerative processes.

of lamellipodia. The main Ca^{2+} source in growth cones that promotes neurite outgrowth may change during development and give rise to changes in the level of Ca^{2+}, leading to activation of different signaling cascades. Ooashi et al. (2005) reported that CICR mediated by type 3 RyR in the growth cone led to an attraction response whereas repulsion was observed in the absence of RyR function. Ca^{2+} release from internal stores *via* IP_3R in growth cones is reported to be an essential event in Ca^{2+}-dependent neurite outgrowth in chick neurons (Takei et al. 1998).

In hippocampal neurons, neurite outgrowth is regulated by a specific family of transient receptor potential channels (TRPC). The mechanism of activation is dependent on activation of PLC by guidance cues, which in turn leads to the production of IP_3 and Ca^{2+} release from ER. Depletion of these stores opens the TRPC and Ca^{2+} influx occurs (Davare et al. 2009).

The explicit role of IP_3Rs in the regulation of neurite outgrowth was also proven using pharmacological blockade of IP_3R function with 2-APB in chick DRG neurons (Iketani et al. 2009). Time-lapse recording showed that after 2-APB applications (100 μM), neurite growth was arrested and neurites were subsequently retracted within 1 h of 2-APB application. No effect of a RyR inhibitor on neurite outgrowth of chick DRG neurons was confirmed. Growth cones exposed to 2-APB also showed a decrease in clustering of neuronal calcium sensor 1 (NCS-1) suggesting that the neurite outgrowth inhibited by 2-APB is linked to a decrease in the specific distribution of NCS-1 clusters. Therefore, it can be considered that NCS-1, which is functionally linked to IP_3R1 in the growth cone, may promote neurite outgrowth. Immunocytochemical analyses have shown co-localization of NCS-1 with IP_3R1 in the central domain of the growth cone (Schlecker et al. 2006). The data suggest that NCS-1 potentially couples to the IP_3R and may act as a positive regulator of neurite outgrowth in chick DRG neurons. Iketani et al. (2009) confirmed that NCS-1 in growth cones plays an important role in the regulation of neurite outgrowth and growth cone morphology. The data suggest that the regulation of neurite outgrowth by NCS-1 may be mediated by calcium signaling associated with IP_3R1.

In pathophysiological conditions, such as mechanical injury, damage, infection or inflammatory processes, the normal outgrowth of axons is disturbed. As mentioned above, processes involved in axon growth, guidance and navigation are abrogated and frequently a "glial scar" is created and referred to as retractive gliosis to separate the lesion from healthy tissue (Fig. 1).

Astrocytes, microglia and glial progenitor cells around the lesion site are activated and increase in number. They express and release various bioactive substances that play important roles in tissue repair processes including inflammation, blood-brain barrier repair and neural protection (Rolls et al. 2009). Above all, there is a major rearrangement of the anatomical structure. Immediately after CNS injury, resident astrocytes become hypertrophic and extend thick

processes and show increased glial fibrillary acidic protein (GFAP) immunoreactivity. Upregulation of GFAP immunoreactivity in astrocytes is observed as early as 1 day after injury and the processes further create a glia limitans enclosing the lesion site or inflammation thereby protecting the tissue from further spread of the lesion (Kawano et al. 2012). Axonal outgrowth in neurons in close vicinity to the scar becomes inhibited and the cells degenerate (Kimura-Kuroda et al. 2010).

In vitro fibrotic scars constructed from primary cultures of neurons growing on primary co-cultures of astrocytes and fibroblasts treated with transforming growth factor β1 (TGFβ1; Kimura-Kuroda et al. 2010) represent a model that shows very similar molecular and biochemical features to actual CNS lesions. Neurons growing in this model avoid the scar, shorten their axons and lose appropriate synaptic plasticity. Although a more sophisticated methodological approach is necessary and TGFβ1 modulation is too complex for studying behavior of neurons, the value of the information gained on the changes in intracellular calcium concentration, IP_3-induced calcium release, CICR or changes in expression of IP_3Rs and RyRs and other calcium transporters may provide more credible information on the response of the neuron to complex pathophysiology that it encounters during the process of neurodegeneration.

Conclusion

In addition to the data discussed above, there is a growing evidence that disturbed regulation of Ca^{2+} transport and individual intracellular calcium transporters within a neuronal cell interferes dramatically with physiological growth, morphology and plasticity of neurons and participates in the initiation and development of severe traumatic neuronal injuries or damages and consequent degenerative processes that result in serious neurological diseases, such as Alzheimer´s, Huntington´s, Parkinson´s disease and others.

To achieve the closest experimental approach to neuronal physiology and pathophysiology and to study the particular role of Ca^{2+} and the involvement and modulation of participating Ca^{2+} transporters within a single neuron, neuronal primary culture models are preferred. It is obvious that such models are distant from the complex disease pathophysiology, however, they may provide a useful tool for studies of regulatory pathways and may help to discover novel potential therapeutic targets. Ca^{2+} transporters, such as IP_3Rs, RyRs, and other Ca^{2+} signaling proteins could be considered as one of those targets.

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