● REVIEW

Neurotoxicity of the pesticide rotenone on neuronal polarization: a mechanistic approach

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Abstract

Neurons are the most extensive and polarized cells that display a unique single long axon and multiple dendrites, which are compartments exhibiting structural and functional differences. Polarity occurs early in neuronal development and it is maintained by complex subcellular mechanisms throughout cell life. A well-defined and controlled spatio-temporal program of cellular and molecular events strictly regulates the formation of the axon and dendrites from a non-polarized cell. This event is critical for an adequate neuronal wiring and therefore for the normal functioning of the nervous system. Neuronal polarity is very sensitive to the harmful effects of different factors present in the environment. In this regard, rotenone is a crystalline, colorless and odorless isoflavone used as insecticide, piscicide and broad spectrum pesticide commonly used earlier in agriculture. In the present review we will summarize the toxicity mechanism caused by this pesticide in different neuronal cell types, focusing on a particular biological mechanism whereby rotenone could impair neuronal polarization in cultured hippocampal neurons. Recent advances suggest that the inhibition of axonogenesis produced by rotenone could be related with its effect on microtubule dynamics, the actin cytoskeleton and their regulatory pathways, particularly affecting the small RhoGTPase RhoA. Unveiling the mechanism by which rotenone produces neurotoxicity will be instrumental to understand the cellular mechanisms involved in neurodegenerative diseases influenced by this environmental pollutant, which may lead to research focused on the design of new therapeutic strategies.

Key Words: *rotenone; environmental pollutants; toxicity; neuronal polarity; RhoGTPase; RhoA; Lfc; Arhgef1*

Introduction

In all vertebrate and most invertebrate animals, the brain is the central organ of the nervous system. In this regard, neurons are the structural and functional units of the central nervous system, exhibiting a highly polarized morphology containing two structurally and functionally domains, called the axon and the dendrites (Barnes and Polleux, 2009; Yogev and Shen, 2017). The establishment and maintenance of neuronal polarity is carried out through several processes, among which we include a diversity of signaling pathways, an asymmetric distribution of cellular structures, and a specialized machinery devoted to the transport of membrane organelles and proteins. The precise regulation of the organization and dynamics of the cytoskeleton is essential for the development of neuronal polarity (Conde and Cáceres, 2009; Bentley and Banker, 2016; Schelski and Bradke, 2017). Dissociated cell culture of neurons is the canonical model for studying neuronal physiology at the single cell level. In this context, cultured hippocampal neurons represent a valid experimental model to unravel the mechanisms involved in the neurotoxicity of certain pesticides, since they undergo a stereotypic differentiation process *in vitro* (Coullery et al., 2016; Sethi et al., 2017).

A multifactorial etiology is attributed to numerous neurodegenerative pathologies such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, which may result from the interaction between environmental factors and genetic predisposition which could lead to disorders on neuronal polarization (Etemadifar et al., 2012; Dardiotis et al., 2013; Marques and Outeiro, 2013; Baltazar et al., 2014). Numerous authors have linked pesticides, including rotenone, with these pathologies; however, their specific effect, as well as the precise mechanisms, are not completely understood. Rotenone, a broad-spectrum pesticide, has been strongly linked to pathophysiological mechanisms implicated in experimental models of human Parkinson's disease (Johnson and Bobrovskaya, 2015).

It has been demonstrated that the subcutaneous administration of rotenone in a dose of 2–3 mg/kg per day during 7 days induces the pathology of Parkinson's disease in Lewis rats (Betarbet et al., 2000). Despite the mechanisms related to the toxic effects caused by rotenone in catecholaminergic neurons were extensively studied (Ren et al., 2005; Ren and Feng, 2007), much less effort has been devoted to analyze the effects of the pesticide on the morphological differentiation of neuron, especially during the development of the establishment of neuronal polarity and the mechanisms associated. Considering this, the present review article summarizes main evidence about the mechanism by which rotenone affects both the development and maintenance of neuronal polarity, pay in attention to new emerging questions and future challenges on the field of neurotoxicity. Literature searches were performed on public databases (PubMed) using the terms "Rotenone neurotoxicity" and "Neuronal polarization".

Mechanism of Rotenone Toxicity in Catecholaminergic Neurons

Rotenone, a potent mitochondrial complex I inhibitor (Xiong et al., 2012), is a hydrophobic compound that easily traverses the blood brain barrier causing disorders in the central nervous system. Numerous studies have shown that rotenone administration (from 0.1 nM up to 10 μM) for a period of 12 hours, to rat embryonic midbrain cultured neurons of 14 days *in vitro* induces selective apoptosis on serotonergic and dopaminergic neurons (Ren et al., 2005; Ren and Feng, 2007). Along this line, a similar result was obtained when SH-SY5Y neuroblastoma cells were incubated for 24 hours with 10 μM rotenone (Wu et al., 2018).

It has been demonstrated that the selective and progressive dopaminergic neurodegeneration provoked by rotenone is highly po-

tentiated by the release of the enzyme NAD(P)H (nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate) oxidase-derived superoxide from the activated microglia (Gao et al., 2003). In catecholaminergic neurons rotenone, at micromolar concentrations, also exerts neurotoxicity by inhibiting complex I of the mitochondrial respiratory chain and by inducing mitochondrial membrane depolarization (Nisticò et al., 2011), leading to oxidative stress mediated by abnormal high levels of reactive oxygen species and nitric oxide (Simon et al., 2000; Circu and Aw, 2010). Subsequently, rotenone activates c-Jun N-terminal kinase 3 (Choi et al., 2010) and p38 mitogen-activated protein kinases/p53 signaling pathway both *in vivo* and *in vitro* (Wu et al., 2013). The activation of p53 induce the translocation of the proapoptotic proteins Bim (Bcl-2 interacting mediator) and Bax from the cytosol to the mitochondria, leading to the mitochondrial release of the proapoptotic factors cytochrome c and Smac (second mitochondria-derived activator of caspases)/DIABLO (direct IAP-binding protein with low PI), which finally promotes the proteolitic cleavage and final activation of caspase 3 and 9 (Ahmadi et al., 2008; Lee et al., 2008; Lin et al., 2012). Rotenone also redistributes dopamine from synaptic vesicles to the neuronal cytosol (Watabe and Nakaki, 2007); due to the inhibition of vesicular monoamine transporter 2 activity (Guillot and Miller, 2009; Choi et al., 2015). In this way rotenone also stimulates reactive oxygen species production and protein carbonylation, as well as decreases the levels of glutathione, the main endogenous antioxidant of cells. Therefore, free dopamine in the neuronal cytoplasm induces the generation of quinones and semi-quinones produced either by dopamine autooxidation or by its oxidation and further enzymatic degradation by the monoamine oxidase (Fornstedt et al., 1990; Chiueh et al., 1993). Secondary metabolites produced by these reactions affect the physiology of proteins. **Figure 1** summarizes the convergence of the rotenone-induced pathways with catecholaminergic-mediated neuronal apoptosis

Rotenone Inhibits Neuronal Polarization in Cultured Hippocampal Neurons

Cultured hippocampal neurons have been extensively used to study neuronal polarization; they undergo a series of morphological changes during differentiation. Initially (*e.g.*, shortly after plating) neurons do not have a discernible polarity displaying a large lamelipodia that in most cases completely surrounds the cell body (stage I); later, lamelipodia collapse, being replaced by a symmetrical array of minor processes (stage 2). Afterwards, one of these minor neurites elongates to develop as the axon (stage 3) (**Figure 2**). One week later, minor neurites are differentiated into dendrites (stage 4), whereas synaptic development emerges after two weeks in culture (stage 5) (Dotti et al., 1988).

Using this model system, morphological analysis has revealed that rotenone administration below the apoptotic range (0.1 μM) for 48 or 72 hours produces a selective and total inhibition of axon formation without affecting the generation of minor neurites (Sanchez et al., 2008). This suggests that rotenone does not prevent initial process outgrowth, but the axonal specification process. This phenomenon will be deeply analyzed in the next sections. On the other hand, the treatment of dopaminergic neurons with 0.1 μM of rotenone during first 24 or 48 hours after plating also selectively reduces the length of the longest neurite of developing neurons (Sanchez et al., 2008). These results suggest that in young developing neurons the pesticide prevents axon outgrowth and elongation. Besides, they are in accordance with studies carried out by Tomaselli et al. (2005) who showed that 10 μM of the pesticide impairs the

extension of neurites in PC12 cells. Furthermore, authors proposed that the developmental effect of the pesticide is specific and may involve the activation of signaling cascades directly related with the development of neuronal polarity.

Rotenone Affects Microtubule and Actin Cytoskeleton Stability During Neuronal Polarization in Hippocampal Neurons

A delicate balance of both negative and positive signals regulates the cellular machinery that specifies which of the minor processes differentiate into an axon. When this equilibrium is broken (by a pro-polarizing signal), a positive feedback cycle is initiated, and a single minor neurite grows faster in order to become an axon. This self-activating system produces strong negative feedback signals directed to the rest of the neurites, preventing the development of additional axons (Arimura and Kaibuchi, 2007; Conde and Cáceres, 2009; Takano et al., 2017). During axonal formation, rigorous cellular mechanisms strictly regulate the dynamics and stability of both the microtubule and actin cytoskeletons, as well as the vesicular transport and membrane addition at sites of active growth (Quiroga et al., 2018). It is of capital importance to emphasize that the cytoskeleton provides a crucial scaffolding to coordinate different signaling pathways to promote the growth of a single axon and numerous dendrites. Currently, it has been well described a four step-process required for the conversion of a minor neurite into an axon: (1) an increase in the amount of plasma membrane addition due to polarized transport and vesicle fusion to the selected neurite; (2) a local enhancement of the activity of signaling molecules, such as Rho GTPases; (3) an increase in actin dynamics on the leading neurite and (4) an increase in microtubule formation at the site of axonal specification (Arimura and Kaibuchi, 2007). Thus, both actin and microtubules are crucial components of the cytoskeleton indispensable for the development of polarity in neurons (Schelski and Bradke, 2017).

Considering the inhibitory role of rotenone on neuronal polarity, we wonder about a putative link between this pesticide and cytoskeleton dynamics. In fact, pioneering studies demonstrated that rotenone binds reversibly to tubulin for preventing microtubule assembly, evaluated on the mammalian cell line CHO (Brinkley et al., 1974) as well as on *in vitro* microtubule formation assays (Marshall and Himes, 1978), both experiments either in the presence or absence of MAPs (microtubule-associated proteins). Subsequently, it was shown that some of the toxic effects of the rotenone, both in non-neuronal and neuronal cells, involve microtubule depolymerization. In this regard, experiments performed on cultured dopaminergic and serotoninergic neurons, rotenone (from 0.1 nM up to 10 μM), produces both microtubule depolymerization and disruption of the vesicular transport through microtubules, triggering the consequent accumulation of dopamine-containing vesicles in the neuronal cell body (Ren et al., 2005; Ren and Feng, 2007). This issue leads to oxidative stress increment and sequentially to dopamine oxidation stored at the cytoplasm that were released from the synaptic vesicles (Ren et al., 2005; Ren and Feng, 2007). Moreover, previous works have shown that rotenone inhibits mitosis and prevents MCF-7 and HeLa cells proliferation by altering microtubule assembly with an half maximal inhibitory concentrations of 0.4 ± 0.1 μM and 0.2 ± 0.1 μM, respectively (Srivastava and Panda, 2007).

At neuronal level, recent studies carried out in cultured hippocampal neurons showed that rotenone-treated neurons exhibit enhanced microtubule dynamics, elevating the chance that these neurons fail to differentiate into an axon due to failures to generate stable microtubules (Bisbal et al., 2018). In agreement with this ob-

servation, the microtubule-stabilizing drug Taxol, partially reverts this inhibitory effect of the pesticide on axonogenesis (Bisbal et al., 2018).

Regulation of actin activity and organization within growth cones is another key event involved in the establishment of neuronal polarity. In this context, it has been demonstrated that application of low doses of cytochalasin D, an actin-depolymerizing drug, favors the penetration of microtubules inside the neuritic tips and consequently leads to the formation of supernumerary axons (Bradke and Dotti, 1999). Results from our group (Sanchez et al., 2008) pointed out that the depolymerization of actin with cytochalasin D reverts the inhibitory effects of the pesticide (0.1 μM) on axonogenesis. Therefore, rotenone may also impair axon formation by stabilizing F-actin at axonal growth cone. In agreement with these results, it has been shown that rotenone, in addition to moderately decreasing ATP levels, may also reduce the actin polymerization rate in GD25 cells (Zeller et al., 2013).

Effect of Rotenone upon GTPases Family

The family of small RhoGTPases is a group of signaling G proteins, whose prototypical members arecell division control protein 42 (Cdc42), Rac1, and RhoA. One of the main roles of this family is regulate the assembly, disassembly, and dynamic reorganization of the actin and microtubule neuronal cytoskeleton (Wojnacki et al., 2014). It is not surprising, therefore, that they play crucial roles in the differentiation, growth, guidance and branching of axons, and any modification (up- or down-regulation) in their activities can affect the establishment of neuronal polarity and consequently cause developmental and neurodegenerative diseases (Antoine-Bertrand et al., 2011; Stankiewicz and Linseman, 2014; Datta et al., 2015).

Three families of regulatory proteins control the RhoGTPases cycle between active and inactive states: (a) guanine nucleotide exchange factors (GEFs), which stimulate Rho activity favoring the exchange of guanosine diphosphate for guanosine triphosphate (GTP); (b) GTPase activating proteins, responsible for stimulating the hydrolysis of GTP causing, in this way, the inactivation of the Rho-GTPases; and (c) The guanine nucleation-dissociation inhibitors that bind to the RhoGTPases and prevent their activity. In their active state, the RhoGTPases bind and modify the activity of downstream effectors regulating, in this way, many cellular events (Cherfils and Zeghouf, 2013). In cultured hippocampal neurons, rotenone (0.1 μM) is able to reduce both Cdc42 and Ras-related C3 botulinum toxin substrate 1 (Rac1) activities, whereas an up-regulating effect over RhoA activity is detected (Sanchez et al., 2008). The initial extension of neurites is not influenced by the suppression of either Rac1 or Cdc42 (Kunda et al., 2001; Sosa et al., 2006). Conversely, the normal activity of Cdc42 and Rac1 is essential for axonogenesis (Kunda et al., 2001; Nishimura et al., 2005; Sosa et al., 2006), therefore the decrease in their activities would prevent axonal formation. On the other hand, the increase in RhoA leads to the stabilization of the F-actin, inhibiting axonal growth (Bito et al., 2000; Conde et al., 2010). These observations could explain why rotenone inhibits axonal growth without affecting the development of minor neurites.

Consistent with this, inhibition of Rho kinase (ROCK) activity, a downstream effector of RhoA, as well as increased Rac1 activation induced by ectopic expression of its GEF Tiam1 (T lymphoma invasion and metastasis 1), reverts the inhibition caused by the pesticide on axon formation in cultured hippocampal neurons. Together, these data indicate that some of the neurotoxic effects of pesticide are linked with an inhibition of actin dynamics *via* modifications of Rho-GTPase activity (Sanchez et al., 2008).

After evaluating rotenone-increased RhoA activity effect (Sanchez et al., 2008), opposed results were obtained by Fujimura and Usuki (Fujimura and Usuki, 2012), which reported that in cortical neurons rotenone (0.1 μM) only decreases the activity of Rac1, without affecting RhoA activity. Strikingly, in this report authors indicate that either *RhoA* knockdown or ROCK activity inhibition in cultured hippocampal neurons, protects neurons from the effect of rotenone, suggesting a role of RhoA signaling on the neurotoxic effect triggered by the agrochemical. Subsequent studies using a FRET (Förster resonance energy transfer)-based second generation RhoA biosensor (Pertz et al., 2006; Fritz et al., 2013) confirmed the increase of RhoA activity, an observation that was experimentally complemented by pull-down assays (Sanchez et al., 2008). Furthermore, results obtained through the use of the Eevee-ROCK FRET biosensor (Li et al., 2017), show that the agrochemical increases the activity of ROCK and that this activation is reduced by Taxol (Bisbal et al., 2018). These results would provide additional evidence of the participation of RhoA in the arrest of axon growth induced by rotenone.

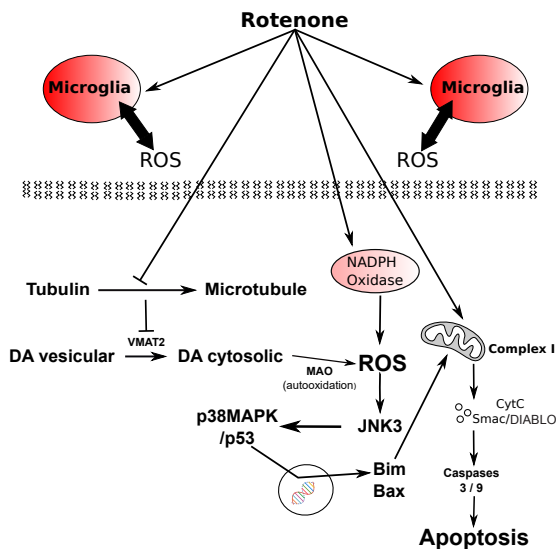## Rotenone Prevents Axonogenesis Through Lfc/RhoA Pathways

So far, more than 80 RhoGEFs have been identified in the human genome (Rossman et al., 2005; Goicoechea et al., 2014). Among them, the most studied RhoA GEFs are Arhgef1 (Rho guanine nucleotide exchange factor 1) and Lfc (GEF-H1). Arhgef1 expression is enhanced in early stages of neuronal development. Both in primary cortical neurons and in Neuro-2a cells, Arhgef1 acts as a negative regulator of neurite outgrowth by regulating F-actin dynamics through RhoA-cofilin pathway (Xiang et al., 2016). Along the same line, Lfc localizes both at the Golgi apparatus and minor neurites growth cone of developing neurons, negatively regulating neurite sprouting and axonal formation through the RhoA signaling pathway (Conde et al., 2010). In this regard, it has been described that inactive Lfc remains attached to microtubules and its activation only occurs after its release, which depends on microtubule depolymerization (Birkenfeld et al., 2008). After this, Lfc switch on and promotes RhoA activity, impairing axonal formation and neurite sprouting (Conde et al., 2010).

A study conducted to analyze a probable interrelation between the pesticide, RhoA activity, Lfc expression and axonogenesis showed that rotenone (0.1 μM) increases Lfc protein levels in cultured hippocampal pyramidal neurons. Moreover, the suppression of Lfc expression, but not of Arhgef1, prevents the inhibition of axonogenesis caused by rotenone (Bisbal et al., 2018). Finally, these results suggest that, in cultured neurons, rotenone induces microtubules destabilization and detachment of Lfc from microtubules, which promotes RhoA and ROCK activation and, sequentially, the inhibition of axonogenesis (**Figure 2**).

A striking aspect that remains to be explored is the effect of rotenone on neuronal development and differentiation *in situ*. Most of the available evidence regarding the effect of rotenone on axon dendrite formation derives from studies in cultured neurons. This is important since at least for certain neuronal types (*e.g.* cortical pyramidal neurons) neuronal polarization *in situ* is different from that observed in culture; for instance, while *in situ*, there is a multipolar to bipolar transition at the time of axon specification, this phenomenon has not been observed in cultured neurons (Funahashi et al., 2014; Hansen et al., 2017). Future analyses of the effect of rotenone on neuronal polarization *in situ* promises great advances on our knowledge of the cellular and molecular mechanisms involved in the neurotoxicity caused by this agrochemical.

Final Conclusions

Considering the progressive increasing incidence of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, as well as multiple sclerosis and other aging-associated neurodegenerative disorders, it is vital to understand the mechanisms by which environmental contaminants can affect the nervous system and increase the risk of producing such pathologies. Here we discuss recent findings on how rotenone exerts neurotoxicity in cultured neurons and more significantly on the cellular mechanism by which the pesticide affects neuronal development. Understanding the molecular mechanisms involved in neurotoxicity caused by environmental factors is crucial both for the early detection of neurodegenerative alterations and for the promising translation of possible therapies, which is the final purpose.

Figure 1 Model illustrating the molecular mechanisms implied in rotenone-induced neurotoxicity in dopaminergic neurons.
Rotenone favors the production of reactive oxygen species (ROS) in the cytoplasm by three different mechanisms that include: 1) By depolymerizing microtubules. When the microtubules are depolymerized, the vesicular transport of dopamine (DA) is interrupted, which results in an increase in the concentration of cytosolic DA and the consequent increase in ROS levels due to autooxidation of the DA caused by monoamine oxidases (MAO). 2) By inhibition of complex I of the mitochondrial chain and 3) By the activation of several ROS-generating enzymes like the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases. It is important to note that rotenone can induce neuronal oxidative stress (OS) through the activation of microglia. The accumulation of ROS leads to OS due to the oxidation of nucleic acids, proteins and lipids that mediate the activation of the signaling cascades that lead to apoptosis. VMAT2: Vesicular monoamine transporter 2; p38MAPK: p38 mitogen-activated protein kinases; JNK3: c-Jun N-terminal kinase 3; Bim: Bcl-2 interacting mediator; CytC: cytochrome c; Smac: second mitochondria-derived activator of caspases; DIABLO: direct IAP-binding protein with low PI.

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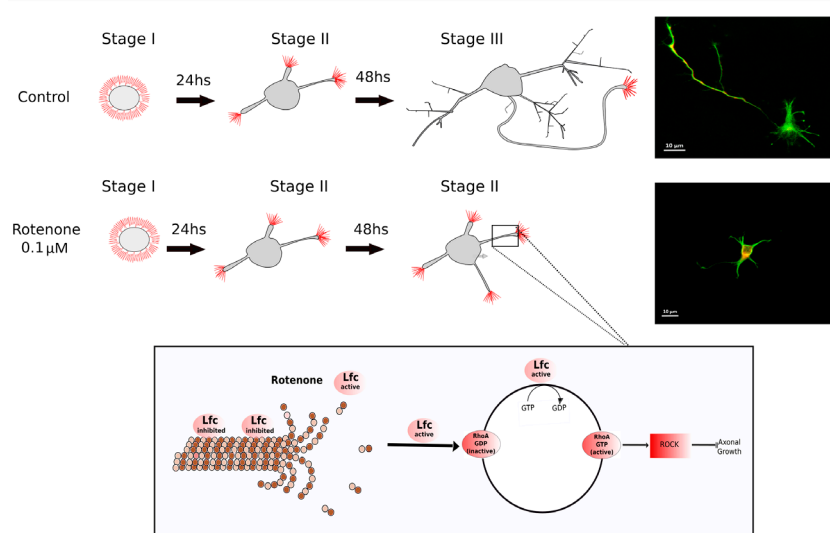
Figure 2 Rotenone inhibits axon growth by an Lfc/RhoA/ROCK *via* in cultured hippocampal neurons.
Rotenone produces a selective and complete inhibition of axonogenesis in cultured hippocampal neurons. The mechanism involved in the inhibition of the axonogenesis implies the stimulation of the RhoA/ROCK pathway resulting from the changes in microtubule dynamics and the simultaneous release of Lfc (GEF-H1), a microtubule-associated guanine nucleotide exchange factor (GEF) specific for RhoA. Micrograph shows control and rotenone-treated 72 hs hippocampal cultured neurons stained with anti tyrosinated tubulin (green) and anti-axonal marker Tau1 (red) antibodies. hs: Hours; GTP: guanosine triphosphate; GDP: guanosine diphosphate; ROCK: Rho kinase.

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