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The Use of the Mitochondrial Toxin 3-NP to Uncover Cellular Dysfunction in Huntington's Disease

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1. Introduction

Degenerative diseases that affect the nervous system are characterized by a progressive alteration of specific neuronal populations and normally end in cell death. Some neurodegenerative disorders exhibit a clear genetic origin, such as in the case of Huntington's Disease (HD); however, most neurodegenerative diseases do not have a genetic cause, suggesting that other mechanisms cause these alterations.

Even though each neurodegenerative disease exhibits specific features, many similarities in the degenerative process are also shown; the study of these similarities could provide ideas for the therapeutic management of such diseases. For example, Parkinson's, Alzheimer's, and Huntington's Diseases exhibit atypical protein assemblies, excitotoxicity, metabolic alterations, oxidative stress and mitochondrial failure (Shoffner et al., 1991; Sims et al., 1996; Kapogiannis and Mattson, 2011). All of these cellular alterations can trigger one or more forms of cell death, namely apoptosis, necrosis and/or autophagy. Normally, excitotoxicity and inflammatory activations are associated with necrosis as a type of neuronal death (Artal-Sanz and Tavernarakis, 2005); the apoptotic type of cell death is often associated with the activation of cysteine protease caspase-3 (Kroemer et al., 1998). However, in post-mortem tissue from HD patients, no clear histological or pathological data are available in support of apoptotic cell death (Vis et al., 2005), although DNA damage in the progression of degeneration was shown to be evident (Brouillet et al., 1999). Also, in animal models of neurodegeneration, oxidative stress promotes apoptotic damage, triggered by the activation of caspases (Burke et al., 1996; Krantic et al., 2005). In neurodegeneration, it is important to understand which type of cell death mechanism is involved however, it is also important to be aware that the speed of cell death process in "sick" neurons is slow (Kanazawa, 2001).

2. Mitochondrial dysfunction

Studies over several decades have documented experimental evidence to support the fact that mitochondrial dysfunction and oxidative stress are part of the cellular mechanisms

underlying neurodegeneration. Mitochondrial dysfunction can occur early on in the pathogenesis of several diseases, including HD (Koroshetz et al., 1997; Jenkins et al., 1993, 1998; Panov et al., 2002; Lin and Beal, 2006).

During mitochondrial respiration, reactive oxygen species (ROS) are produced as by products of respiratory chain activity; their overproduction generates oxidative stress, and is a hallmark of neurodegenerative disorders. The respiratory chain is composed of five complexes: complexes I and II collect electrons from the catabolism of fats, proteins and carbohydrates and transfer them to co-enzyme Q10, complex III and complex IV. Importantly, complexes I, III and IV utilize the energy produced by the electron gradient generated by pumping protons across the inner mitochondrial membrane; this proton gradient is used by complex V to condense Adenosine diphosphate (ADP) and inorganic phosphate into Adenosine-5'-triphosphate (ATP). Any alteration in the mitochondrial complex is expected to produce ATP deficiency (Fridovich, 1999), release of cytochrome *c* which activates the intrinsic pathways of neuronal death and the activation of caspases related to apoptotic damage (Maciel et al., 2004).

3. Huntington Disease

Huntington's Disease, also called Huntington's chorea because of the presence of rapid and incessant choreic movements accompanied by cognitive and psychiatric alterations, is an inherited neurodegenerative disease that affects cell projections in a specific region of the brain known as the nucleus striatum. This neurodegenerative illness was described in 1872 by George Huntington, although some features that resemble HD had already been described earlier (Walker, 2007).

The symptoms manifest in the third or fourth decade of life in most cases, and progression of the illness is slow (15 to 20 years). In comparison to other neurodegenerative diseases, the development of HD is associated with the mutation of a single gene called *Interesting Transcrip 15* (IT15), or the *Hd* gene. The mutation originates the expansion of the CAG nucleotide repeats in a single protein called huntingtin; wich is called mutant huntingtin (mhhtt), when the CAG expansion is present (Taylor et al., 2002). This single mutation produces diverse cellular, physiological and anatomical changes; however, the cellular mechanisms underlying HD neurodegeneration are not yet fully understood.

A number of studies have shown that mitochondria from HD patients and animal models are damaged (Jenkins et al., 1993), suggesting that disturbances in the cellular metabolism of HD patients originate via mitochondrial dysfunction (Beal, 2005). Deficits in energy metabolism become manifest in the pre-symptomatic and symptomatic HD brain and peripheral tissues (Kuhl et al., 1982; Mazziotto et al., 1987; Grafton et al., 1990; Koroshetz et al., 1997; Lodi et al., 2000). In particular, factors related to mitochondrial functioning seem to underlie the selective vulnerability of striatal cells (Saft et al., 2005; Seong et al., 2005); for example, the reduction of the chaperone, protease and intramembrane mitochondrial molecule Omi/HTr2 (Inagaki et al., 2008), and alterations in oxidative phosphorylation functioning in general (Pickrell et al., 2011). Post-mortem studies on HD brain tissue showed decreased activity in complexes II, III and IV of the mitochondrial respiratory chain (Gu et al., 1996). Also, animal models of HD showed deficits in mitochondrial respiration (Browne et al., 1997); for example; the systemic administration of mitochondrial toxins or inhibitors

generated striatal pathology and movement disorders such as chorea and dystonia, which resemble HD (Ludolph et al., 1991; Browne, 2008). In fact, accidental ingestion of the irreversible mitochondrial inhibitor 3-nitropropionic acid (3-NP) was found to cause striatal degeneration and the HD phenotype. Moreover, the systemic administration of 3-NP caused striatal cell loss and movement alterations in rats (Beal et al., 1993) and primates (Palfi et al., 1996; Dautry et al., 2000). However, an analysis of unbiased gene expression showed that changes in energy metabolism in mhtt of transgenic mice compared to 3-NP treated animals were different: while 3-NP affected mitochondrial pathway gene expression, the effects of mhtt on metabolism were extramitochondrial (Lee et al., 2007), which suggested that mitochondrial toxins such as 3-NP do not quite cause HD pathology (Olivera, 2010). However, mhtt generates mitochondrial dysfunction in HD (Grunewald & Beal 1999) and reductions in ATP generation (Seong et al., 2005), and it also alters Ca^{2+} buffering (Reddy et al., 2009) and mitochondrial trafficking (Li et al., 2010). Therefore, irrespective of whether mhtt impacts mitochondria directly or secondarily, the repercussions of mitochondrial dysfunction are devastating to cells and may underlie the disruptions in numerous cellular processes, resulting in HD pathogenesis. As a result, the identification of respiratory chain changes in complex II of respiratory chain in HD post-mortem brains led to the use of mitochondrial complex II inhibitors to generate toxicity models that replicate aspects of HD striatal pathology *in vivo*. Thus, studies on mitochondrial toxins are relevant and important for understanding defects in cellular metabolism and the energetic pathogenesis of Huntington's Disease.

4. 3-Nitropropionic acid (3-NP)

3-NP is a highly specific, time dependent and irreversible inhibitor of succinate dehydrogenase (SDH) and the Krebs cycle (Alston et al., 1977). The levels of inhibition of this enzyme by 3-NP correlate well with the levels of inhibition of the tricarboxylic acid cycle (Henry et al., 2002).

4.1 Changes in the central nervous system

3-NP treatment was found to cause striatal degeneration in rodents (Gould and Gustine 1982; Gould et al., 1985; Beal et al., 1992; Brouillet et al., 2005). In non-human primates, 3-NP produced cognitive deficits similar to those displayed by frontal-type and abnormal choreiform movements, followed by evident striatal degeneration (Palfi et al., 1998; Brouillet et al., 1999; Dautry et al., 2000).

It is known that 3-NP imitates the symptoms of dystonia, glutaric aciduria, Leber's disease and HD (Novotny et al., 1986; Janavs and Aminoff 1998; Strauss and Morton, 2003), but after discovering that the accidental ingestion of 3-NP (He et al., 1995; Ming, 1995) caused damage that was concentrated in the striatum, 3-NP was used in experimental models to study the cellular mechanisms underlying striatal neural degeneration (Alexi et al., 1998).

The initial studies suggested that excitotoxicity plays a central role in the physiological and cellular effects of 3-NP on striatal degeneration (Hamilton and Gould, 1987; Novelli et al., 1988; Zeevalk and Nicklas, 1990) because of the presence of massive glutamatergic afferents in the nuclei (Di Figlia et al., 1990); also, metabolic insults were suggested as playing a part in the mechanistic damage (Browne et al., 1997; Brouillet et al., 1999). Chronic treatment with 3-NP in rats produced astrogliosis and selective degeneration of medium-sized spiny

neurons, similar to the neurochemical and histological pathology observed in post-mortem HD tissue. Interestingly, 3-NP treatment was found to retain terminals from large cholinergic interneurons and NADPH-diaphorase-positive aspiny interneurons (Beal et al., 1993; Brouillet et al., 1999).

The type of death triggered by 3-NP treatment depends on how the toxin was administered: intraparenchymal applications induce ischaemic injury features while intraperitoneal applications induce striatal degeneration, which shows more of an HD phenotype (Borlongan et al., 1997a). Another concern about the 3-NP pharmacological model of HD is related to the variations in cellular damage, which depend on whether a study is carried out *in vitro* or *in vivo*, whether rats or mice are used, whether 3-NP is administered intrastratially or intraperitoneally, whether the treatment is acute or chronic, and whether low, sub-toxic or toxic concentrations are provided (Brouillet et al., 1999, 2005).

3-NP administered by subcutaneous (s.c.) or intraperitoneal (i.p.) injections is more toxic in rats than in mice. In rodents, the toxicity of 3-NP depends on the strain (Brouillet et al., 2005). Fisher rats, for example, are more susceptible to 3-NP toxicity than Sprague-Dawley, Wistar and Lewis strains (Ouay et al., 2000), and C57BL/6 and Balb/c mice are more resistant to 3-NP toxicity than 129SVEMS and FVB/n mice (Gabrielson et al., 2001). The strain-dependent differences observed following 3-NP intoxication are probably related to differences in elimination/detoxification of the compound (Ouay et al., 2000). Vulnerability to different agents is not restricted to animal strains; it occurs in all human groups and is due to genetic variations that give rise to different responses to drugs (Weinshilboum et al., 2003; Weinshilboum and Wang, 2006), so we need to be cautious when generalizing about the results obtained among different strains.

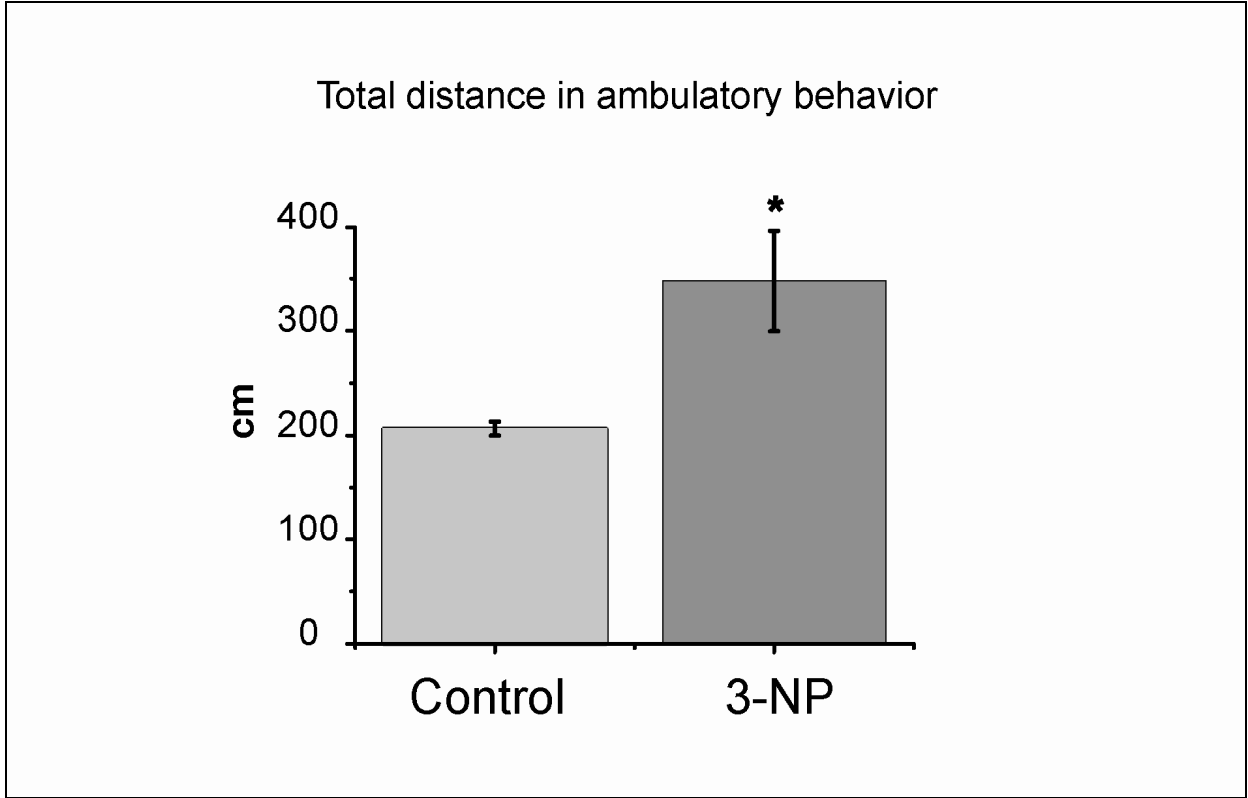
Other important factors are the age and gender of the animals used in experimental protocols (Brouillet et al., 1993). Interestingly, female rats are less sensitive to 3-NP than males, which suggests that oestrogen protection can affect the degree of sensitivity (Nishino et al., 1998; Mogami et al., 2002).

The method used for 3-NP delivery also influences the physiological effect; acute treatments of a single i.p. dose of 3-NP were found to lead to striatal degeneration within 6-12 h after injection (Alexi et al., 1998; Brouillet et al. 1999). Sub-chronic treatments consisting of daily repeated i.p. injections led to striatal degeneration over a few days (Beal et al., 1993; Schulz et al., 1996; Guyot et al., 1997). Chronic treatments (of more than 5 days up to 4 weeks) with the continuous systemic administration of 3-NP using subcutaneously implanted osmotic minipumps also produced striatal degeneration. Besides the mode of delivery, the treatment dose concentration also has an impact on 3-NP toxicity.

Depending on the time period over which 3-NP is administered, and the dose administered, rodents treated with 3-NP exhibit HD-like motor disorders with hyperkinetic and hypokinetic symptoms (Borlongan et al., 1997), and rats were shown to be more sensitive to the effects of 3-NP than mice (Brouillet, 2005). In rats, the administration of 3-NP (10 mg/kg i.p.) over several days was found to induce the onset of hypokinetic symptoms (Guyot et al., 1997), while its administration in two individual doses caused hyperkinetic symptoms (Borlongan et al., 1997b). However, besides the mode of delivery, the treatment dose also has an impact on 3-NP toxicity, where a 3-NP concentration of ~20 mg/kg was found to induce the expression of an HD behavioural phenotype after two injections. Nevertheless,

these animals did not display extra-striatal lesions, which are frequently observed in the initial stages of HD (Beal et al., 1993; Guyot et al., 1997; Borlogan et al., 1997b). The chronic administration of low doses of 3-NP (~10 mg/kg, per day) for more than 3 weeks was found to induce sustained metabolic alterations and some other cellular features exhibited in HD patients, but did it not cause clear dyskinetic movements resembling chorea (Borlogan et al., 1997a, b; Brouillet et al., 1999).

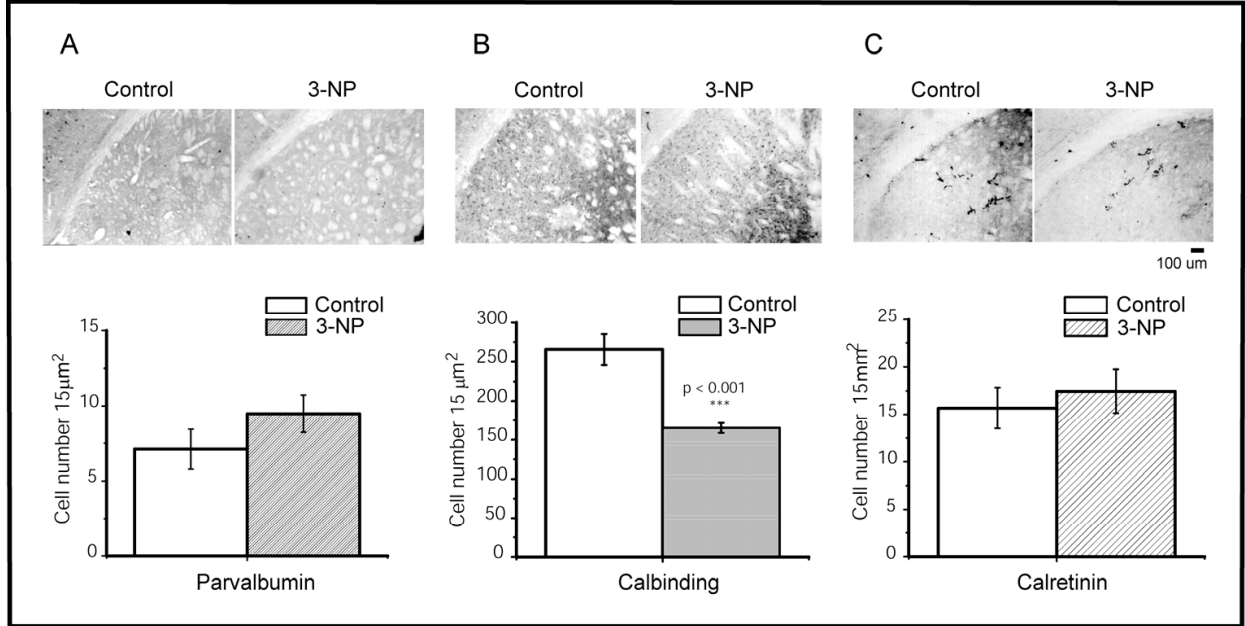
Since there are differences in the response to 3-NP treatment among different animal strains (Ouary et al., 2000), we designed an administration plan of low concentration doses of 3-NP (15 mg/kg, i.p.) over a sub-chronic period (5 days) in C57BL/6 mice, which are known to be more resistant to 3-NP toxicity; this advantage enabled us to observe histopathological changes that mimic those found in the initial steps of the illness (Rodriguez et al., 2010), plus motor alterations such as orofacial dyskinesias and claspings behaviour (Hernández-Echeagaray et al., 2011), and spontaneous behaviours that resemblance the HD phenotype. Figure 1, shows that mice treated with low dose concentration of 3-NP displayed motor hyperactivity, as evaluated in open field tests. Hyperkinetic symptoms are exhibited in the initial steps of striatal damage in animals treated with 3-NP, whereas the hypokinetic phenotype develops later during striatal deterioration (Borlongan et al., 1997b).



The graph shows the effects of 3-NP (15 mg / kg, per 5 days) in the spontaneous ambulatory motor behaviour of mice, scored as the total distance during 10 minutes period, evaluated in the open field test (Versadata, 3.02-1E7E software, Accuscan Instruments, INC.). There was a significant increase in locomotor activity in the 3-NP treated group (t-test, *p=0.0213). Arithmetic means and standard errors are plotted.

Fig. 1. 3-NP increases the ambulatory behavior evaluated in open field test.

We are especially interested in discerning the cellular events that take place during the beginning of the neurodegenerative process; the understanding of early dysfunctions will help in the planning of therapeutic strategies to reduce or delay cellular damage. The neuronal damage caused by low doses of 3-NP administration is restricted to striatal calbindin-positive cells (Fig. 2), leaving sparing parvalbumin and calretinin positive interneurons as previously suggested (Ferrante et al., 1987a, 1987b, Kowall et al., 1987, Massouh et al., 2008). Cellular alteration can also be initiated by caspase 3-dependent apoptosis, although necrotic cell death is also present (Rodriguez et al., 2010).



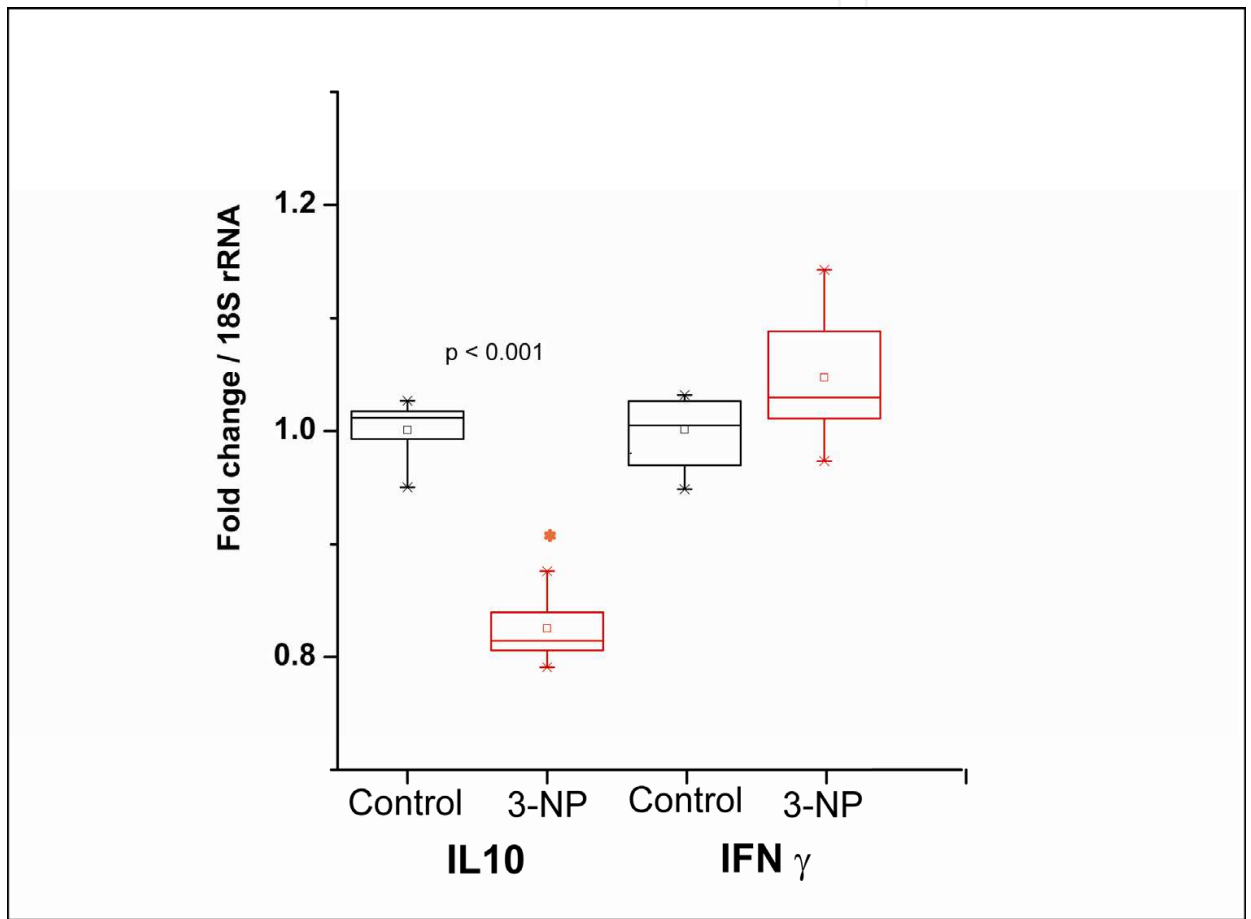
Top: light micrographs of calcium binding proteins immune localization in the neostriatal tissue. A, illustrates cells immune positive to parvalbumin. Cells were homogenously distributed in the striatum in both groups. The staining of immune positive cells was located in the soma. Graph in the bottom shows that there were no differences between groups in the number of cells expressing parvalbumin ($t_{35} = -1.519$, $p = 0.138$). B illustrates striatal cells that were immune positive to calbindin. Calbindin positive cells were homogenously distributed along the striatum in both groups and its localization was concentrated in the cell soma. Graph in the bottom shows that in tissue of 3-NP treated group, immune positive cells were significantly reduced in comparison to control group ($T_{99} = 2073.5$, $p < 0.001$). C illustrates calretinin immune positive cells. These cells were localized in dorsal striatum and staining was observed in cell body, dendrites and axonal ramifications. Interestingly tissue from 3-NP treated mice exhibited a decrease in cell ramifications but as shown in the bottom graph, the immune stained cell number did not change between groups ($T_{82} = 1686.5$, $p = 0.523$). Scale bar is 100 μm.

Fig. 2. 3-NP significantly decreases medium spiny neurons identified with the calcium binding protein calbindin.

3-NP treatment induces the abnormal production of reactive oxygen species (ROS), as well as highly reactive molecules derived from the formation of nitric oxide (NO). Succinate dehydrogenase inhibition interferes with the electron transport cascade and oxidative phosphorylation, which results in a decrease in ATP production and a cellular energy deficit (Jana et al., 2001; Lunkes et al., 2002). In studies by our group, the systemic administration of low sub-chronic doses of 3-NP did not produce a significant augmentation of NO in the brain (Rodriguez et al., 2010); however, NO and lipid peroxidation (LPO) increased in

skeletal muscle (Hernández-Echeagaray et al., 2011). Previous reports looking at the involvement of NO in the toxicity of 3-NP did not draw any clear conclusions, but it has been suggested that 3-NP acts as an NO donor, increasing the levels of nitro anions (Jana et al., 2001; Lunkes et al., 2002).

Increases in NO and LPO along with signs of necrosis that manifest at the ultra-structural level in 3-NP-treated animals, like cellular oedema, are suggestive of the inflammatory process. Gene expression of the anti-inflammatory cytokine Interleukin-10 (IL-10) was found to significantly decrease whereas expression of the inflammatory cytokine Interferon-gamma (IFN γ) increased, indicating that, in low doses, 3-NP reduces the activation of anti-inflammatory cytokines (Fig. 3). This may have negative effects to prevent cellular damage.



PCR products of IL-10 and IFN γ in striatum of control and 3-NP treated groups are displayed. Data were analyzed by measuring continuously gene-specific PCR products and differences were assessed with the 2 $_{-\Delta\Delta CT}$ method. Data are presented as the fold increase in gene transcripts normalized to the 18S rRNA expression and relative to the control. IL-10 expression was significantly reduced in the 3-NP group (t13 = 12.904, p<0.001). IFN γ exhibited a no significant increase in the 3-NP treated group (t16= 0.232, p= 0.819).

Fig. 3. Expression of striatal IL-10 and IFN γ mRNA by real time RT-PCR.

Cells obtain energy from oxidative phosphorylation and from glycolysis; the glycolytic enzyme GAPDH has been implicated in neuronal degeneration (Taylor et al., 2002; Huntington's Disease Collaborative Research Group, 1993) and a reduction in GAPDH

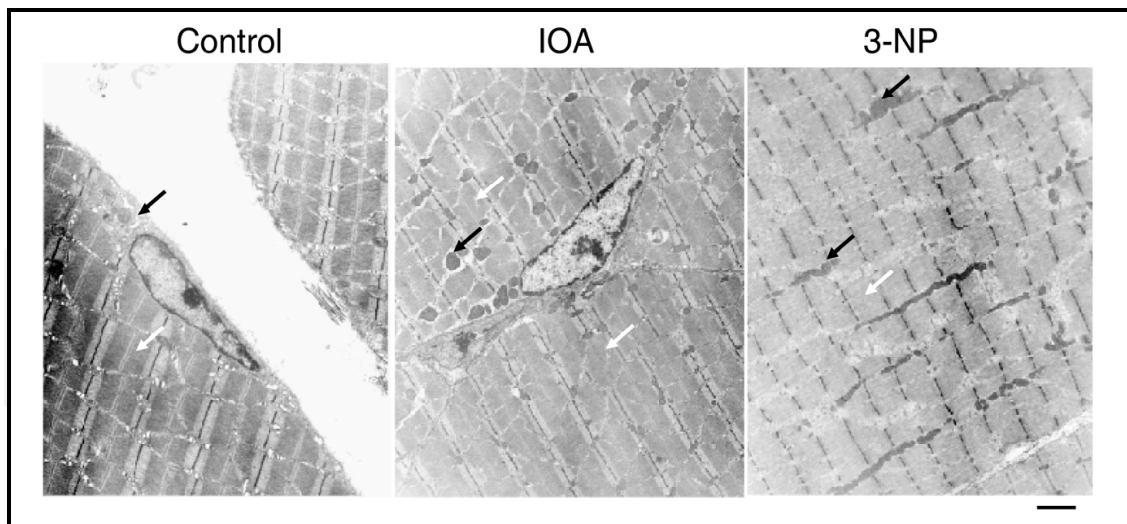
activity was demonstrated in HD patients and in a transgenic mouse model of HD (Burke et al., 1996; Matthews et al., 1997). Some studies have suggested that mitochondrial failure is secondary to striatal damage in HD (Lee et al., 2007), and then another energy source that may fail and generate damage is glycolysis. Both oxidative phosphorylation and glycolysis can be involved in the pathogenesis of neural degeneration in the striatum. However, inhibition of the glycolytic enzyme GAPDH was found to cause apoptotic damage, which is independent of the activation of caspase 3 (Rodriguez et al., 2010). Hence, alterations in glycolysis may be a critical point in neuronal death, but its inhibition activates different cellular signals than oxidative phosphorylation.

4.2 Changes in the periphery

Any deficit in energy metabolism can damage the entire physiology of an organism. Muscles metabolism is essential for locomotion and heat production (Zierath and Hawley, 2004). Patients afflicted by HD experience many deficits (Bradshaw et al., 1992; Aron et al., 2003; Abbruzzese et al., 2003), and weight loss is a characteristic feature (Robbins et al., 2006; Ciammola et al., 2011; Mochel and Haller, 2011). Transgenic mice model in an HD exhibited protein inclusions (Sathasivam et al., 1999) and mhtt aggregation in skeletal muscle (Ribchester et al., 2004). The motor impairments displayed by patients and animal models could result from central neurodegeneration, but also from alterations in muscle metabolism, reflecting peripheral disturbances as a general metabolic failure. Where metabolic collapse occurs in an organism, this could partly clarify the muscle alterations and body weight loss documented in HD patients (Stoy and McKay, 2000; Robbins et al., 2006; Ciammola et al., 2011) and transgenic HD mice (Sathasivam et al., 1999; Ribchester et al., 2004; She et al., 2011) and the alterations caused by metabolic alterations (Simoneau et al., 1995; Petersen et al., 2004).

Initially, it was thought that 3-NP did not produce major peripheral effects (Hamilton and Gould 1987). Until now, most of the studies that were carried out using 3-NP as a tool to investigate neurodegeneration focused on evaluating central damage (Beal et al., 1993; Borlongan et al., 1997; Brouillet, et al., 1999, 2005), even though in a number of them 3-NP was systemically administered. However, Gabrielson (et al., 2001) showed that 3-NP induced modifications in cardiac muscle physiology.

In addressing the possible changes that 3-NP might generate outside of the brain, our group documented modifications in skeletal muscle after uncoupling oxidative metabolism with 3-NP in low sub-chronic doses. Our hypothesis was that abnormal mitochondrial functioning during first stages of neurodegenerative disease is responsible for body weight loss in patients afflicted with HD or illness where there is a metabolic dysfunction (Stoy and McKay, 2000; Robbins et al., 2006). The systemic administration of low doses of 3-NP altered enzymatic activity in muscle, as well as the organization of the sarcomere (Hernández-Echeagaray et al., 2011), suggesting that energy failure exacerbates metabolic activity in the whole organism, producing high metabolic demands to counteract the failure of bioenergetics. When comparing muscle modifications in animals where glycolysis was inhibited, we found that iodoacetate IOA also alters the ultrastructure of the gastrocnemius muscle; this disorganization was more pronounced in animals that were treated with 3-NP (Fig. 4).



Electron microscopy of gastrocnemius muscle from control, IOA, and 3-NP treated animals. Control muscle shows normal sarcomere organization (white arrow) and mitochondria morphology (black arrow). However, sarcomere of IOA and 3-NP treated groups was altered; also the number of mitochondria and mitochondria morphology were modified. In particular muscles from 3-NP treated group were most affected than those from the IOA group. Micrographics magnification is 7000X. Scale bar is 500 nm.

Fig. 4. Metabolic uncoupling induces ultra structural modification in the mouse gastrocnemius muscle.

Oxidative phosphorylation or metabolic failure may generate an increase in energy consumed by muscles, as has been suggested in several degenerative disorders where alterations in oxidative phosphorylation and metabolism are compromised (Ristow, 2004). It is important to mention that in models of food restriction or malnutrition, the size and body weight of animals were affected before the nervous system became involved in the behavioural phenotype (Woodall et al., 1996; Clapham, 2004). Being aware of the peripheral and central damage due to metabolic dysfunctions might help in the clinical management of the early stages of the disease. For example, treatments designed to improve energy metabolism might modify the course of the illness and delay the progression of the disease.

5. Conclusions

The goal of this chapter was to illustrate the fact that 3-NP in low sub-chronic doses induces cellular alterations that emulate the damage exhibited in the striatum and muscle of patients afflicted by HD in the early stages of the illness. Even though 3-NP does not reproduce all signs and symptoms displayed in HD patients, it is true that it has helped in the understanding of the cellular physiology of animal models where a general failure in bioenergetics has been presumed.

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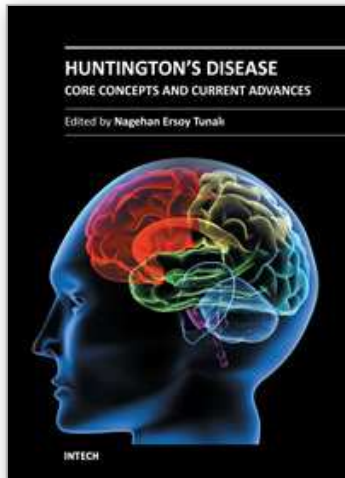
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Huntington's Disease is one of the well-studied neurodegenerative conditions, a quite devastating and currently incurable one. It is a brain disorder that causes certain types of neurons to become damaged, causing various parts of the brain to deteriorate and lose their function. This results in uncontrolled movements, loss of intellectual capabilities and behavioural disturbances. Since the identification of the causative mutation, there have been many significant developments in understanding the cellular and molecular perturbations. This book, "Huntington's Disease - Core Concepts and Current Advances", was prepared to serve as a source of up-to-date information on a wide range of issues involved in Huntington's Disease. It will help the clinicians, health care providers, researchers, graduate students and life science readers to increase their understanding of the clinical correlates, genetic aspects, neuropathological findings, cellular and molecular events and potential therapeutic interventions involved in HD. The book not only serves reviewed fundamental information on the disease but also presents original research in several disciplines, which collectively provide comprehensive description of the key issues in the area.

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