THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 15, pp. 10736–10741, April 12, 2013

Calcium, Bioenergetics, and Neuronal Vulnerability in Parkinson's Disease*

Published, JBC Papers in Press, October 19, 2012, DOI 10.1074/jbc.R112.410530

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The most distinguishing feature of neurons is their capacity for regenerative electrical activity. This activity imposes a significant mitochondrial burden, especially in neurons that are autonomously active, have broad action potentials, and exhibit prominent Ca^{2+} entry. Many of the genetic mutations and toxins associated with Parkinson's disease compromise mitochondrial function, providing a mechanistic explanation for the pattern of neuronal pathology in this disease. Because much of the neuronal mitochondrial burden can be traced to L-type voltage-dependent channels (channels for which there are brain-penetrant antagonists approved for human use), a neuroprotective strategy to reduce this burden is available.

Neurons, Electrical Excitability, and Bioenergetics

Parkinson's disease (PD)[2] is a disease of neurons, but not all neurons are affected by the disease. The pathological hallmarks of PD are 1) the presence of proteinaceous intracellular depositions called Lewy bodies (LBs) or Lewy neurites (LNs) and 2) frank neuronal loss (1). In general, these two signs of the disease are correlated, although not perfectly. If LB/LN deposition is used to quantify pathology, <1% of the neurons in the brain appear to be affected at mid-stages of the disease, so although PD is a disease of neurons, only a subset of neurons are at risk.

What then are the phenotypic features of neurons at risk? A cardinal feature of neurons is their electrical excitability. Neurons use a steep electrochemical gradient across their plasma membrane to integrate incoming chemical signals from other neurons and to pass the outcome of this computation on to other neurons. Excitability and synaptic transmission between neurons depend upon the maintenance of electrochemical gradients for Na^+, K^+, Ca^{2+}, and Cl^- across the plasma membrane. A major challenge to these gradients is the action potential, a regenerative event that transiently opens membrane channels that allow ions to redistribute. In most neurons, the action potential or spike depends upon the opening of voltage-dependent channels that are selectively permeable to Na^+ ions, allowing positively charged Na^+ ions to move from the extracellular space into the cytosol. This redistribution of charge pushes the transmembrane potential from relatively negative membrane potentials to near 0 mV. This depolarization causes voltage-dependent channels that are selectively permeable to K^+ ions to open, resulting in the movement of positively charged K^+ ions in the opposite direction: from the cytosol to the extracellular space, re-establishing the potential gradient. This sequence of events requires that the concentration of Na^+ ions be low in the cytosol, but the concentration of K^+ ions needs to be high.

Another cation that crosses the plasma membrane during spikes is the Ca^{2+} ion. In most neurons, voltage-dependent Ca^{2+} channels are opened only by strong depolarization during the action potential. With repolarization of the membrane, these channels close slowly, creating a period during which the driving force for influx of Ca^{2+} is large and the conductance remains high. This makes the total Ca^{2+} influx during a spike very sensitive to spike duration. Neurons that need to spike at high frequencies typically restrict Ca^{2+} entry by keeping spikes very brief (<1 ms). Also, neurons often express fixed Ca^{2+}-buffering proteins (2), like parvalbumin, in addition to Ca^{2+}-signaling proteins (3) to help manage Ca^{2+}.

Exchangers and pumps are responsible for maintaining the electrochemical gradients for Na^+, K^+, Ca^{2+}, and Cl^-. These transmembrane proteins fall into two broad categories. The first comprises pumps that rely upon ATP to drive the movement of ions. Pumps that fall into this category include the Na^+/K^+-ATPase, the plasma membrane Ca^{2+}-ATPase, and the smooth endoplasmic reticulum Ca^{2+}-ATPase. The proteins in the second category utilize the energy stored in an existing electrochemical gradient to move ions. A good example of this type of protein is the Na^+/Ca^{2+} exchanger, which, under physiological conditions, uses the Na^+ gradient to move Ca^{2+} ions out of the cytosol. Together, this combination of pumps and exchangers maintains the transmembrane ionic gradient for cations. Ca^{2+} that is not pumped back out of the neuron rapidly is sequestered in intracellular organelles, including lysosomes (4, 5) and the endoplasmic reticulum (6). High affinity ATP-dependent transporters move Ca^{2+} from the cytoplasm into these organelles.

Although the molecular events coupling ion movement to ATP hydrolysis are still not fully understood, the thermodynamics of ion movement are worth considering because they establish lower limits on the cost of pumping. For Na^+ and K^+ ions, the concentration differences maintained across the plasma membrane are similar, being 10–30-fold. In contrast, the concentration difference for the Ca^{2+} concentration is ~20,000-fold, being 2 mM in the extracellular space and ~100 nM in the intracellular space. Because the free energy change needed to move an ion from one compartment to another depends upon the logarithm of the concentration ratio between the two compartments, Ca^{2+} should be ~8 times more ener-

* This work was supported, in whole or in part, by National Institutes of Health Grants NS047085, RR025355, and HL35440. This work was also supported by grants from the Hartman Foundation and the United States Army Medical Research and Materiel Command. This article is part of the Thematic Minireview Series on Calcium Function and Disease.

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[2] The abbreviations used are: PD, Parkinson's disease; LB, Lewy body; LN, Lewy neurite; DA, dopamine; SNc, substantia nigra pars compacta; VTA, ventral tegmental area; DMV, dorsal motor nucleus of the vagus; LC, locus ceruleus; RN, raphe nuclei; BF, basal forebrain; ROS, reactive oxygen species; DHP, dihydropyridine.

getically expensive than one Na^+ ion (~4 times greater on a per charge basis). The Na^+/K^+-ATPase extrudes three Na^+ ions and takes up two K^+ ions per ATP molecule consumed. The plasma membrane Ca^{2+}-ATPase pumps one Ca^{2+} molecule out for each ATP consumed. Overall, it takes about one ATP molecule for each Ca^{2+} ion and one ATP molecule for every three Na^+ ions, making Ca^{2+} more energetically expensive than Na^+.

The At-risk Neuronal Phenotype

Although all neurons maintain steep electrochemical gradients across their plasma membrane and spike, not all neurons show signs of pathology in PD. As a consequence, vulnerability in PD must depend upon some constellation of features that define the specific neuronal phenotype, *i.e.* something more specific than just being a neuron. One way to determine that at-risk neuronal phenotype is to characterize neurons affected by the disease in terms of common features. The neurons with the best documented vulnerability are dopamine (DA)-releasing neurons in the substantia nigra pars compacta (SNc). The cardinal motor symptoms of PD, including bradykinesia, rigidity, and resting tremor, are clearly linked to the degeneration and death of these neurons (7). There are only a few tens of thousands of these neurons out of the billions in the brain.

Why do these neurons exhibit LB/LN pathology and die? One possibility is their distinctive physiological phenotype. Adult SNc DA neurons are autonomous pacemakers exhibiting slow broad spikes and lacking significant intrinsic Ca^{2+}-buffering capacity (8–12). Pacemaking is necessary to maintain a basal DA tone in target structures, like the striatum; without it, movement ceases. Although most neurons rely exclusively on channels permeable to Na^+ to drive pacemaking, SNc DA neurons also engage L-type channels with a $Ca_v1.3$ pore-forming subunit, leading to elevated intracellular Ca^{2+} concentrations (10, 13–16). In adult SNc DA neurons, the currents that flow through these channels are of sufficient magnitude to sustain a membrane potential oscillation when voltage-dependent Na^+ channels are blocked with tetrodotoxin (8, 11). Ventral tegmental area (VTA) DA neurons, which also are slow pacemakers but do not manifest these Ca^{2+} oscillations, have much lower $Ca_v1.3$ Ca^{2+} channel density (17) and express high levels of the Ca^{2+}-buffering protein calbindin (18). VTA neurons have a significantly lower risk of degeneration in PD (18, 19).

A second possibility is that oxidation of cytosolic DA (and its metabolites) leads to the production of cytotoxic free radicals (20). However, there are reasons to doubt whether this type of cellular stress *alone* is responsible for PD pathology. For example, there is considerable regional variability in the vulnerability of DA neurons in PD, with some DA neurons in the brain being devoid of pathology (19, 21–24). Moreover, many of the neurons showing signs of pathology in PD do not use DA as a transmitter (*e.g.* cholinergic neurons in the dorsal motor nucleus of the vagus (DMV)). Finally, L-3,4-dihydroxyphenylalanine administration (which relieves symptoms by elevating DA levels in PD patients) does not accelerate disease progression (25), suggesting that DA itself is not a significant source of reactive oxidative stress, at least in the short term. Sulzer and co-workers (26) recently reported that cytosolic DA concentrations are greater in SNc DA neurons than in neighboring VTA neurons because the former utilize Ca^{2+} entry through L-type channels to stimulate DA synthesis. This led to early toxicity in SNc DA neurons during L-3,4-dihydroxyphenylalanine loading, which was abolished by antagonizing L-type Ca^{2+} channels, suggesting that differences in calcium signaling might lead to SNc-specific toxicity arising from excessive DA synthesis (26). Nevertheless, taken together, the available data do not make a compelling argument that DA itself is the principal culprit in PD.

What about at-risk neurons outside of the mesencephalon? The best characterized pathology in PD is found in neurons in the DMV, in the locus ceruleus (LC), in the raphe nuclei (RN), in the gigantocellularis nucleus, in the tuberomammillary nucleus of the hypothalamus, in the olfactory bulb, and in the basal forebrain (BF) (1, 27). These neurons do not share a common neurotransmitter. DMV and BF neurons are cholinergic, LC neurons are noradrenergic, and RN neurons are serotonergic. However, these neurons do seem to share physiological features. All of these nuclei are dominated by spontaneously active neurons, which often have prominent transmembrane Ca^{2+} currents (28–38). Although the spontaneous activity in BF neurons is extrinsically generated (39), the activity of neurons in the LC, DMV, tuberomammillary nucleus, and RN is known to be intrinsically generated, as in SNc DA neurons. Many of these have broad (>2-ms half-width) action potentials like SNc DA neurons. Furthermore, where it has been examined (LC, BF, and DMV), these neurons have low intrinsic Ca^{2+}-buffering capacity (40, 41).[3]

Collectively, these neurons form a distinct subset of pacemaking neurons in the brain. Autonomous pacemakers can be divided into fast and slow spiking classes. Fast autonomous pacemakers, like globus pallidus and substantia nigra pars reticulata GABAergic neurons, spike at rates above 20 Hz at physiological temperature (42, 43), have very brief action potentials (<1 ms), and robustly express Ca^{2+}-binding proteins (44). These neurons show no obvious pathology in PD. The slow pacemakers (the neurons at risk in PD) spike at much lower frequencies (~2–5 Hz) but have broad spikes (>2 ms) that favor Ca^{2+} entry.

Thus, at this point, a reasonable hypothesis is that neurons at greatest risk in PD share a common physiological phenotype: sustained spontaneous spiking, broad spikes, prominent Ca^{2+} currents, and low intrinsic Ca^{2+}-buffering capacity. This phenotype is very rare in the diencephalon and telencephalon but is more common in the phylogenetically older parts of the brain, like the mesencephalon and brainstem.

Why Is This Phenotype a Risk Factor?

Because of their high basal energy demand, neurons depend upon mitochondria to supply the ATP necessary for survival. Under normal conditions, glucose is metabolized by neurons to produce ATP. Glycolysis results in the generation of 2 mol of ATP/mol of glucose converted to pyruvate. However, when the pyruvate is transported into the mitochondria and further oxidized by the TCA cycle and the electron transport chain, the

[3] D. J. Surmeier and P. T. Schumacker, unpublished data.

total yield of ATP increases to 36 mol/mol of glucose. The dependence of a cell on mitochondrial oxidative phosphorylation can be evaluated with genetic models. For example, deletion of TFAM (transcription factor A, mitochondrial) abolishes mitochondrial gene transcription, undermines the stability of mtDNA, and leads to a progressive decline in mitochondrial ATP production even though the mitochondrial mass may be increased. Deletion of TFAM in SNc DA neurons leads to respiratory chain deficiency, cell loss, and impaired motor function (45). This observation is consistent with an abundance of literature demonstrating that mitochondrial toxins taken up by dopaminergic neurons lead to their demise (46). That said, the dependence upon mitochondria for survival is probably a universal feature of neurons, not just SNc DA neurons (47).

What differentiates neurons at risk in PD? The metabolic demands posed by maintaining transmembrane ionic gradients underlying excitability led Nicholls (48) to postulate that neurons are at risk in neurodegenerative disease because they have a modest bioenergetic or respiratory reserve. This reserve is defined as the difference between the maximum capacity for ATP generation by oxidative phosphorylation and the basal consumption of ATP. The smaller this respiratory reserve, the greater the likelihood that episodic demands on metabolism, like exposure to a toxin or bursts of spiking, will cause cellular ATP levels to fall and create a bioenergetics crisis that could lead to failure of membrane pumps or other ATP-dependent processes. Persistent loss of membrane potential leads to massive Ca^{2+} influx and cell death (49, 50). Clearly, slow pacemaking neurons with broad action potentials, sustained Ca^{2+} influx, and low intrinsic Ca^{2+}-buffering capacity (like SNc DA neurons) would be at the bad end of the respiratory reserve distribution of neurons, putting them at risk for deficits of this kind.

A second possibility is that the increased metabolic demands on at-risk neurons give rise to an increase in the basal level of oxidant stress in their mitochondria. Mitochondria have long been known to generate reactive oxygen species (ROS) (51). The transfer of four electrons to O_2 at Complex IV yields H_2O, but on occasion, single electrons are captured by O_2 at proximal sites in the chain, yielding superoxide anion, a free radical. Sites implicated in that process include Complexes I and III and possibly certain dehydrogenases of the TCA cycle (52). Complex II can potentially generate superoxide when electron flux through its B, C, or D subunit is disrupted by mutations, pharmacological inhibition, or genetic deletion (52). To protect against the detrimental effects of matrix oxidant stress, a set of "antioxidant" systems is expressed in the mitochondria to degrade ROS. Manganese superoxide dismutase is a nuclear gene imported into the matrix, where it dismutes superoxide anions into hydrogen peroxide (H_2O_2). H_2O_2 is then degraded by glutathione peroxidase and peroxiredoxins III and V, which are also expressed in the matrix. A pool of GSH resides in the mitochondria and is used by the reductase systems to clear H_2O_2 and lipid hydroperoxides. The resulting GSSG is subsequently re-reduced to GSH by glutathione reductase, which utilizes NADPH. The resulting $NADP^+$ is then re-reduced to NADPH by dehydrogenases in the TCA cycle, such as isocitrate dehydrogenase-2. Under physiological conditions, the rate of superoxide production is balanced by the rate of clearance, preventing the accumulation of superoxide/H_2O_2, thereby protecting the mitochondria from oxidative damage.

Superoxide can affect protein function by reacting with iron-sulfur centers or heme groups, whereas peroxides can react with lipids, proteins, and DNA. To the extent that these mitochondrial ROS can impair mitochondrial function, they threaten the function of cells with limited respiratory reserve by further limiting the ability of the mitochondria to respond to increases in metabolic demand.

The factors regulating the generation of superoxide from the electron transport chain are not fully understood. Some observations suggest that mitochondrial membrane hyperpolarization can augment ROS generation. In Complex I, reducing equivalents from NADH are transferred to ubiquinone, generating ubiquinol, a membrane-soluble electron carrier that delivers a pair of electrons to Complex III. Complex II links the TCA cycle to the electron transport chain by oxidizing succinate to fumarate, also delivering the electrons to Complex III via ubiquinol. In isolated mitochondria given succinate to supply electrons to Complex II in the absence of ADP, the membrane potential increases. As Complex I is reversible, hyperpolarization can create a retrograde electron flux from ubiquinol to NADH. This is associated with a large increase in ROS production from Complex I (53). It can be inhibited by a modest drop in membrane potential, as the reverse flux is favored only when the membrane is highly polarized. Importantly, this phenomenon is generally limited to isolated mitochondria given succinate as the sole electron source and is otherwise not active in mitochondria respiring on mixed substrates.

Other mechanisms of ROS production in the mitochondrial matrix may relate to electron flux, with high rates of activity correlating with increased ROS generation. In that regard, a high rate of metabolic activity by the cell would be associated with a high rate of ATP hydrolysis, with a corresponding increase in the return of ADP + P_i to the oxidative phosphorylation system. The resulting increase in ATP synthase activity would tend to decrease the membrane potential, thereby increasing the flux of electrons down the chain, increasing the likelihood that some electrons will be captured by O_2 before they reach cytochrome oxidase (54, 55).

Genetic defects in the expression or structure of electron transport complex subunits also can enhance the generation of superoxide. For example, in humans who are heterozygous for the B, C, or D subunit of succinate dehydrogenase (Complex II), cells can undergo somatic cell loss of heterozygosity, leading to the deletion of that subunit from the cell (56). The A subunit of Complex II contains the succinate dehydrogenase catalytic site, whereas the B, C, and D subunits are responsible for transferring the electrons to ubiquinone. If the B, C, or D subunit function is disrupted while the A subunit is still expressed, transfer of the electrons to ubiquinone cannot occur, and they become stranded on the flavin group, which provides a ready source of electrons for superoxide generation. Accordingly, Guzy *et al.* (57) found that knockdown of the B subunit was associated with an increase in the basal ROS generation by mitochondria, whereas knockdown of the A subunit was not. The complete loss of Complex II function in a cell leads to inhibition of elec-

tron transport coupled to proton extrusion and thus a loss of oxidative phosphorylation. Survival of the cell then depends entirely on ATP production by glycolysis, resulting in the release of lactic acid. Ironically, in that state, the mitochondria shift to become consumers of ATP, which is taken up from the cytosol and used to maintain the mitochondrial membrane potential through reverse operation of Complex V.

Recent work by our group has shown that pacemaking in SNc DA neurons does indeed create a basal mitochondrial oxidant stress (58). In this study, we utilized a transgenic mouse that expressed a mitochondria-targeted redox-sensitive variant of GFP (mito-roGFP) (59) under the control of the tyrosine hydroxylase promoter. The use of roGFP allowed us to quantitatively estimated the mitochondrial matrix redox state, something not possible with conventional redox probes. Using two-photon laser scanning microscopy to monitor mito-roGFP in brain slices from young adult mice, we found that pacemaking created an oxidant stress in the mitochondria that was specific to the vulnerable SNc DA neurons and not apparent in neighboring VTA DA neurons. This difference in oxidant stress level was virtually eliminated by antagonizing L-type Ca^{2+} channels, as well as by limiting mitochondrial calcium uptake using the ruthenium-based compound Ru360. These findings suggest that the increased mitochondrial activity caused by the entry of extracellular Ca^{2+} and the subsequent increase in Ca^{2+} uptake by mitochondria leads to an increase in the basal generation of oxidant stress in the mitochondria of SNc DA neurons.

That oxidant stress engages defenses manifested by transient mild mitochondrial depolarization or uncoupling. The mild uncoupling was not affected by deletion of cyclophilin D, which is a component of the permeability transition pore, but was attenuated by genipin and purine nucleotides, which are antagonists of cloned uncoupling proteins. Knocking out *DJ-1* (also known as *PARK7* in humans and *Park7* in mice), a gene associated with an early-onset form of PD, increased oxidation of matrix proteins specifically in SNc dopaminergic neurons. The results with the *DJ-1* knock-out, showing that the impact of *DJ-1* deletion depends upon a physiological phenotype that engages mitochondrial oxidant defenses, provide an example of how mutations in a widely expressed gene can affect a select subpopulation of neurons.

Can oxidant stress (regardless of how it is generated) and mtDNA damage induced by oxidants explain the selective loss of SNc DA neurons in PD? Deletion mutations in mtDNA can arise when H_2O_2 in the matrix introduces double-strand breaks; accordingly, the frequency of these mutations is decreased in hearts of mice expressing mitochondrial catalase (69). In two studies published simultaneously, Bender *et al.* (60) and Kraytsberg *et al.* (61) assessed the abundance of mtDNA deletions, as opposed to point mutations, in SNc neurons from human subjects. The number of mtDNA deletions was significantly greater in SNc neurons from older compared with younger subjects. By contrast, undetectable levels of deletions were found in the cerebral cortex, cerebellum, and dentate nucleus of aged individuals (61). This indicates that cell-specific differences in the occurrence of deletions can exist, consistent with the cell-specific manifestations of PD. Comparisons among single neurons from the same subject revealed that one neuron might contain no deletions whereas another would contain multiple copies of a single species, indicating that they originated from a single initial mutant DNA copy that was clonally amplified in that cell (61). The mechanisms responsible for the clonal expansion of a single deletion mutant copy of mtDNA are not fully known, but the consequences are profound.

mtDNA mutations can affect cell survival by causing bioenergetic failure. When the abundance of damaged mtDNA is below a critical level, the normal copies of mtDNA are adequate to supply the organelle with the proteins needed for ATP production. However, when the abundance of the mutant form exceeds a critical level (typically 60%), the phenotypic defect in function becomes evident. Accordingly, Kraytsberg *et al.* (61) found that the presence of mtDNA deletions correlated strongly with the absence of cytochrome oxidase immunostaining. mtDNA encodes three critical subunits of cytochrome oxidase, so these findings suggest that mtDNA deletions may be responsible for the development of respiratory insufficiency in the affected cells. Like other neurons, SNc DA neurons depend on mtDNA for survival (45), so the accumulation of mutations can lead to the development of a bioenergetic deficiency that becomes lethal over time. As discussed above, loss of electron transport chain function can turn mitochondria into ATP consumers, further stressing bioenergetic status. In PD, it is conceivable that the progressive clonal expansion of the deletion mutant could occur over many years, resulting in the progressive demise of DA cells.

Does the accumulation of deletion mutations in mtDNA of SNc neurons lead to an amplification of ROS generation that triggers a "vicious cycle" in this population of cells? Deletions of mtDNA can lead to the expression of truncation mutant proteins or to the complete loss of subunit expression. When one subunit of a mitochondrial complex is genetically deleted, the remaining subunits are still expressed and degraded, which can lead to the generation of an unfolded protein response in the matrix (62, 63). By augmenting mitochondrial and possibly endoplasmic reticulum stress responses, deletion mutations in mtDNA can thereby amplify oxidant stress in mitochondria and other cellular compartments, pushing the cell already burdened with enhanced oxidant stress even closer to the edge. Hence, the cellular consequences of mtDNA damage may depend importantly on the nature of the mutation.

How do SNc neurons in aged subjects compare with those from individuals with PD? Bender *et al.* (60) found that the degree of mtDNA deletions was somewhat higher in neurons from affected individuals compared with aged-matched controls. This was associated with a significantly greater proportion of cytochrome oxidase-deficient cells, compatible with the idea that bioenergetic crisis could be responsible for the progressive cell loss. Consistent with the study of Kraytsberg *et al.* (61), this group detected clonal expansion of the unique species of mtDNA deletions in individual SNc neurons, indicating that these are indeed somatic mutations. By contrast, high levels of mtDNA deletions were not detected in the hippocampus.

If this type of functional design puts neurons at risk, why did evolution not eliminate it? On average, symptoms in PD appear after 6 decades of life, which is well past the reproductive period

and well past the normal life expectancy until recently. Hence, the evolutionary pressure to change this design is minimal.

Are L-type Ca^{2+} Channels a Viable Therapeutic Target?

L-type Ca^{2+} channels might be a viable therapeutic target in the early stages of PD. These channels are antagonized by orally deliverable dihydropyridines (DHPs) with good brain bioavailability that have a long record of safe use in humans. Is there evidence that DHP L-type channel antagonist use might work in humans to prevent or slow PD? Three major epidemiological studies unequivocally suggest that use of brain-penetrant DHPs diminishes the risk of developing PD (64–66). This linkage is especially surprising given the short period of treatment for inclusion and the lack of DHP potency at $Ca_v1.3$ L-type Ca^{2+} channels. What is less clear is whether DHP use can slow the progression of the disease once diagnosed, as the clinical signs of PD become apparent only when dopaminergic cell loss is extensive. Recent work suggests not (67), possibly because the existing drugs are weak $Ca_v1.3$ channel antagonists or, at this advanced stage, other factors, like inflammation, begin to alter disease progression (68). In the absence of biomarkers that predict disease onset well in advance of the transition to the symptomatic phase, the only obvious way of addressing this question is by early treatment of those harboring mutations that increase disease risk, but this is a small (<10%) fraction of the PD population, making statistical power problematic. The only alternative at this point is a carefully designed prospective clinical trial in early-stage patients with isradipine, the DHP with the highest $Ca_v1.3$ channel affinity and good pharmacokinetics.

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