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Review Article

Neuronal Mitochondrial Trafficking Impairment: The Cause or a Consequence of Neuronal Dysfunction Caused by Amphetamine-Like Drugs

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Abstract Drugs of abuse cause a variety of complex neuronal events at the cellular level, including changes in membrane excitability and neurotransmission, activation of complex signaling pathways, altered synaptic physiology and structural changes, and drug-evoked synaptic plasticity and neurotoxicity, which mediate both acute- and long-lasting effects and addiction. Neuronal mitochondria are highly dynamic organelles that, by undergoing fusion and fission events, are efficiently translocated along the neuronal processes, frequently changing direction, pausing or switching to persistent docking. The neuronal integrity and functionality are dependent upon the proper maintenance of a healthy mitochondrial population and their efficient distribution. There is a general consensus that mitochondrial-dependent pathways can provide a major understanding concerning pathological processes underlying neurotoxicity of drugs of abuse. As such, it is plausible to consider that alterations on mitochondrial trafficking may be key players on the neuronal effects mediated by these drugs. This work aims to provide a comprehensive and up-to-date review of the data linking mitochondrial trafficking impairment to amphetamine-like drugs, and, thus, contribute to a better understanding of their neuronal effects. Additionally, new research data describing alterations in neuronal mitochondrial trafficking for 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy") conjugated metabolites 5-(glutathion-*S*-yl)-*N*-methyl-α-methyldopamine [5-(GSH)-N-Me-α-MeDA] and 5-(*N*-acetylcystein-*S*-yl)-*N*-methyl-α-methyldopamine [5-(NAC)-N-Me-α-MeDA] are also provided.

Keywords drugs of abuse; mitochondria; neuronal mitochondrial trafficking; neurotoxicity

Although the brain constitutes only 2% of the body weight, it consumes approximately 20% of the energy produced in the body. Most brain energy is generated by mitochondria [mitochondria are thought to produce more than 90% of the cellular adenosine 5′-triphosphate (ATP)], highly efficient organelles in utilizing oxygen and substrates, mainly derived from glucose, to produce cellular energy in the form of ATP, via oxidative phosphorylation [42, 55].

In the nervous system, neurons are extremely polarized cells in which mitochondria, organelles usually generated at the soma, are delivered through the neuronal processes to their final destination, the synaptic terminals. Owing to their unique metabolic requirements, these areas do not display a uniform mitochondrial distribution, which implies that the energy production must be spatially matched to local energy usage [53]. On the other hand, though mitochondrial biogenesis may occur locally in the axon, the generation of new organelles mainly occurs within the cell body [2, 63]. Additionally, as mitochondrial degradation ensues in the cell body, dysfunctional mitochondria need to return to the soma for an efficient degradation through the autophagy-lysosomal system (mitophagy) [3]. As such, neurons require highly efficient mechanisms for mitochondrial trafficking regulation, from and to the cell body, to enable the rapid redistribution of mitochondria to different areas, in order to supply distant and metabolically demanding sites, such as synapses, nodes of Ranvier, and active growth cones. A schematic representation of the mitochondrial transport along neuronal axons and dendrites is illustrated in Figure 1.

In mammalian cells, mitochondrial movement is mainly regulated by motor and adaptor proteins through

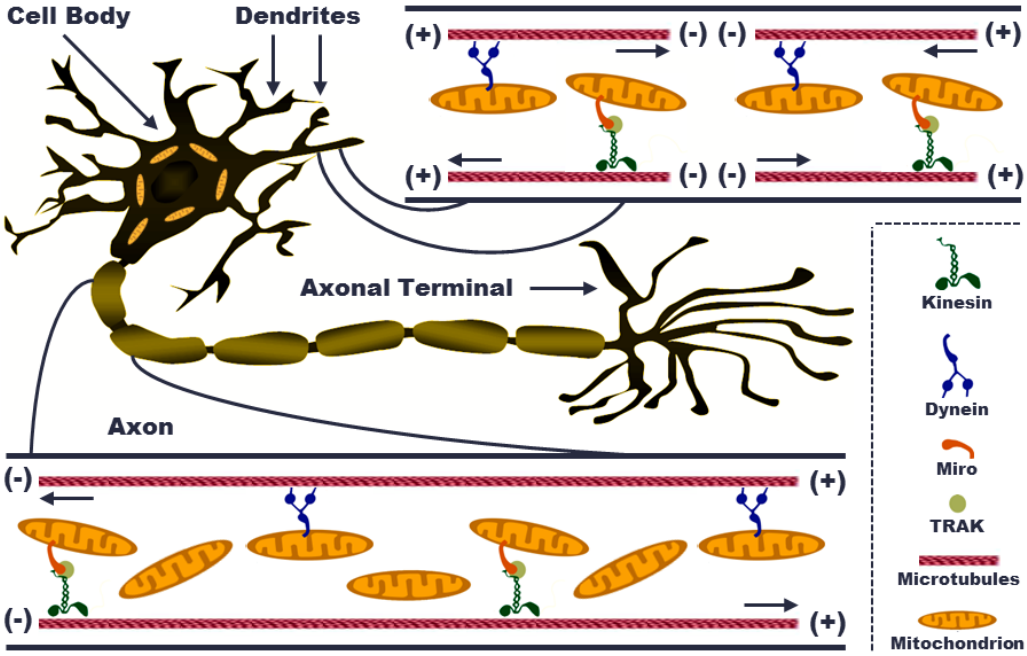

Figure 1: Mitochondrial trafficking in neurons. In axons, microtubules are uniformly organized with the "plus" ends (+) facing toward the axonal terminals and the "minus" ends (-) toward the cell body. However, the organization of microtubules in dendrites show mixed orientation. Polarity and organization of microtubules in axons and dendrites are critical for the targeted transport of synaptic cargoes and organelles by microtubule-associated motor proteins. Whereas kinesin motors are mostly "plus" end-directed, cytoplasmic dynein travels toward the "minus" ends of microtubules. As such, in the axonal compartment, kinesin motors mediate anterograde transport of mitochondria and dynein drives retrograde mitochondrial transport. In dendrites, both kinesin and dynein motors may drive mitochondria in both anterograde and retrograde directions, depending on the microtubule polarity.

microtubules' boundaries [43,51,53]. Microtubules, the main component of the cytoskeleton, are formed by polymers of α- and β-tubulin, which are polarized structures with the "plus" end toward the terminal and the "minus" end toward the cell body. Dendrites, however, present a mixed polarity [49,50]. Cytoskeletal filaments are linked to microtubule-associated proteins (MAPs), such as Tau protein, enabling their stabilization. The kinesin-1 motor family of proteins, also called KIF5, mediates microtubule "plus" end-directed anterograde axonal transport of mitochondria [28,29]. KIF5 functions are supported by adaptor proteins, including *Drosophila* Milton orthologous TRAK1 and TRAK2 [9], linked to the outer mitochondrial membrane Rho guanosine 5′-triphosphatase (GTPase) proteins Miro1/2 [25,27], which act as a calcium sensor [44, 59]. Two additional motor adaptor proteins, syntabulin [11, 41] and fasciculation and elongation protein-ζ1 (FEZ1) [26, 30], have been also described to mediate kinesin-dependent neuronal mitochondrial movement. Microtubule "minus" end-directed retrograde axonal transport of mitochondria is mediated by cytoplasmic dynein motors [28], assisted by the protein dynactin, a multisubunit complex necessary for dynein activity [34]. Biochemical evidence revealed an interaction between dynein motors and the TRAK/Miro complex [56]. Further, potential TRAK-binding partners were identified, such as several components of dynein and dynactin complexes, including dynein heavy-chain 1, dynein light-chain 1 (LC8), p150Glued, and p50/dynamitin [56], thus proposing a regulation of dynein-mediated retrograde mitochondrial transport by TRAK adaptor proteins. Syntaphilin, a neuron-specific and axon-targeted protein, acts as a "static anchor" for docking axonal mitochondria, thereby controlling their axonal movement and density [32].

Since transport events are tightly regulated in response to changes in local energetic state and metabolic demand, mitochondrial trafficking requires the existence of highly coordinated mechanisms to regulate the movement of these organelles along neuronal processes, and their docking to supply specific biological needs. Cytosolic calcium [44,59], MAPs [40,58], and phosphatase and tensin homolog-induced putative kinase 1/Parkin [60,62] are, perhaps, the best characterized regulatory mechanisms of neuronal mitochondrial trafficking. Nevertheless, additional pathways/mechanisms have been emerging, including mitochondrial calcium [16], mitofusin 2 (Mfn2) [45,46] or histone deacetylases [18,33], among others.

The apparent relevance of impaired neuronal mitochondrial trafficking in the context of drugs of abuse-induced neurotoxicity was firstly provided by Callahan and coworkers [12]. In rats, 3 weeks after 3,4-methylenedioxymethamphetamine (MDMA, 20 mg/kg, subcutaneous, twice daily, for 4 days) or fenfluramine (10 mg/kg, intraperitoneal, four times, two-hour interval) administration, the authors found a marked reduction in anterograde transport of labeled material to various forebrain regions known to receive 5-hydroxytryptaminergic innervation. Furthermore, these transport reductions were closely associated with lasting decrements in 5-hydroxytryptaminergic axonal markers [serotonin (or 5-hydroxytryptamine) and 5-hydroxyindoleacetic acid content], thus suggesting a role for impaired axonal transport in developing neurotoxic effects [12]. Nevertheless, in that study, no differentiation between neuronal mitochondrial trafficking and transport of other intracellular organelles or materials was established, not allowing, thus, a clear appraisement of the real impact of these drugs on neuronal mitochondrial movement.

Further in vivo studies sustained a decreased expression of the microtubule-associated protein Tau in mice's ventral midbrain 24 h after methamphetamine administration (45 mg/kg, subcutaneous) [61]. MDMA was, as well, reported to cause Tau phosphorylation in mice's hippocampus (acute MDMA administration of 50 mg/kg, intraperitoneal, or a six-day treatment intraperitoneally with 30 mg/kg) [10] and to disrupt microtubular system in 5-hydroxytryptaminergic axons of rat's frontal cortex (15 mg/kg, intraperitoneal) [1], adding considerable evidence that these drugs of abuse may impair neuronal mitochondrial trafficking.

Following these preliminary data, recent studies conducted by our research group constituted the first direct evidence that MDMA affects neuronal mitochondrial trafficking [8,7]. In one of these studies, following a short exposure period of 90 min, MDMA (1.6 mM) was shown to reduce the overall mitochondrial movement along axonal processes of cultured mice's hippocampal neurons, in a time-dependent fashion.

Microtubule-associated protein Tau, by assembly and maintenance of microtubules, plays a crucial role on neuronal mitochondrial trafficking control [36,40,58]. Additionally, Tau function is largely dependent on its phosphorylation status [40,52]. Therefore, according to the observations indicating increased Tau phosphorylation in mice's hippocampus following MDMA exposure [10], this study also tested the role of Tau protein on MDMA's mitochondrial phenotype. By using Tau-null hippocampal neurons, a partial dependence on Tau protein was observed in MDMA-induced mitochondrial transport arrest [7]. Additionally, it was also revealed that MDMA triggered Tau phosphorylation at Thr181 residue and that the main kinase for Tau protein, glycogen synthase 3β (GSK3β), was involved, as the overexpression of a GSK3β-kinase dead construct, GSK3β Aln9, reduced MDMA-induced mitochondrial trafficking alterations [7].

A functional link between mitochondrial neuronal trafficking control and intracellular calcium levels has been also established, in which the outer mitochondrial membrane protein Miro, by acting as a calcium sensor, plays a crucial role on mitochondrial trafficking control [44, 48,59]. Despite this, by transfecting hippocampal neuronal cultures with wild-type Miro1 protein or a mutant form (Miro1 ΔEF) lacking the EF hand calcium-binding domains, our research group revealed an independence on Miro1 regulatory functions, through cytosolic calcium, in MDMA-induced mitochondrial trafficking impairment [7].

Mitofusins 1 and 2 are guanosine 5′-triphosphatases (GTPases) anchored to the outer mitochondrial membrane, which by forming homo- or hetero-protein complexes allow mitochondrial tethering and fusion [37]. Dynamin-related protein 1 (Drp1), a member of the dynamin family of GTPases, mediates mitochondrial fission [54]. Some observations have indicated that a correct balance between fusion and fission generates discrete mitochondria that may be transported over long distances [2]. Despite this, other functional connections between mitochondrial fission/fusion events and transport have been suggested. With regard to fission, inhibition of the profission protein Drp1 greatly reduced the number of mitochondria in synaptic terminals, thus suppressing synaptic formation and function [38,39,57]. On the other hand, in neurons, lack of the profusion protein Mfn2 was associated with decreased number of mitochondria within their highly branched dendrites [17] and impaired mitochondrial transport in axons [45]. Furthermore, overexpression of an Mfn2 R94Q, a Charcot-Marie-Tooth 2A mutant protein with impaired fusion [23] and transport properties, disrupted axonal transport of mitochondria in both anterograde and retrograde directions [45,46], thus suggesting that uncontrolled fission may result in decreased mitochondrial movement. In our previous work [7], following the observations that MDMA-induced mitochondrial trafficking impairment in hippocampal cultured neurons was accompanied by increased fragmentation of axonal mitochondria, overexpression experiments with wild-type and mutant proteins involved in mitochondrial fusion/fission events were employed. It was revealed that the overexpression of wild-type Mfn2 partially recovered the MDMA's mitochondrial transport phenotype, though Mfn2 R94Q did not [7]. Since fully functional Mfn2 was required to reverse the MDMA-mediated mitochondrial trafficking effects, these results indicated that MDMA might reduce Mfn2 functions, resulting in uncontrolled fission and consequent mitochondrial transport impairment.

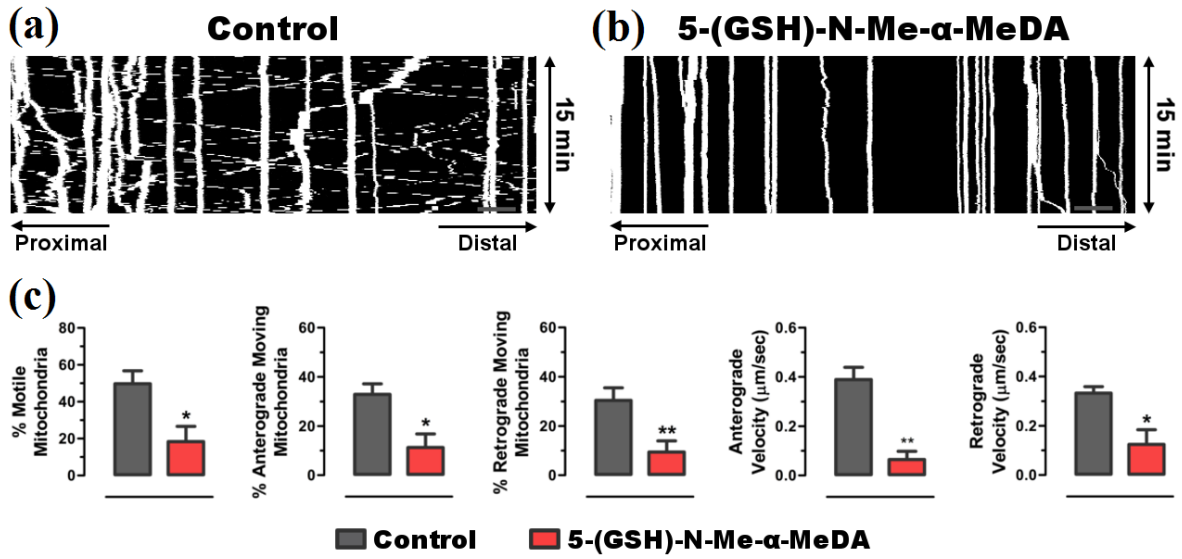

Figure 2: Mitochondrial neuronal trafficking impairment triggered by the MDMA's metabolite 5-(GSH)-N-Me-α-MeDA. (a), (b) Representative kymographs of mitochondrial movement in a single axon under control conditions (a) or after 90 min of exposure to 400 μM of the MDMA's metabolite 5-(GSH)-N-Me-α-MeDA (b). (c) Graphical representation of the percentage of motile mitochondria and velocities, measured in kymographs obtained before and after 5-(GSH)-N-Me-α-MeDA exposure for 90 min. Data represent mean $\pm$ SEM of 7 different axons (neurons), from 5 independent experiments ($^{*}P < .05$, $^{**}P < .01$ 5-(GSH)-N-Me-α-MeDA vs. control, Mann-Whitney's test). Scale bar: 10 μm.

Subsequent experiments overexpressing Drp1 K38A, a dominant-negative mutant Drp1 construct that lacks fission properties, partially recovered the MDMA's mitochondrial transport phenotype. These results further supported the notion that MDMA's mitochondrial trafficking phenotype resulted from a compromised mitochondrial fusion with consequent unrepressed fission.

Since MDMA and its metabolites have been shown to coexist in the brain following peripheral administration of MDMA [20,24,31], further studies of neuronal mitochondrial trafficking were accomplished with MDMA's metabolites 5-(glutathion-*S*-yl)-*N*-methyl-α-methyldopamine [5-(GSH)-N-Me-α-MeDA] and 5-(*N*-acetylcystein-*S*-yl)-*N*-methyl-α-methyldopamine [5-(NAC)-N-Me-α-MeDA] (materials and methods are available as Supplementary Material). The MDMA's metabolite *N*-methyl-α-methyldopamine (N-Me-α-MeDA) is a major hepatic catecholic metabolite in humans, which undergoes further phase II and phase III metabolism. Conjugated MDMA's metabolites can cross the blood-brain barrier and, thus, promote several brain actions [5,6,13]. As shown in Figures 2–3 and Supplementary Movie S1, control neurons exhibit a prominent mitochondrial trafficking, with an average of about 60% of motile mitochondria, in both anterograde and retrograde directions, at a velocity of about 0.4 μm/s. Exposure to MDMA's metabolites 5-(GSH)-N-Me-α-MeDA (Figure 2) or 5-(NAC)-N-Me-α-MeDA (Figure 3 and Supplementary Movie S2), at the concentration of 400 μM for 90 min, dramatically reduced the percentage of motile mitochondria and velocity, in both anterograde and retrograde directions. These data, in accordance with previous published results [4,5,6,14,15], support a major role for metabolism in MDMA-induced neuronal effects.

To better understand the role of metabolism in MDMA's neuronal effects, a further study combined parent compound MDMA and 6 of its major in vivo metabolites, α-methyldopamine (α-MeDA), N-Me-α-MeDA, 5-(glutathion-*S*-yl)-α-methyldopamine [5-(GSH)-α-MeDA], 5-(GSH)-N-Me-α-MeDA, 5-(*N*-acetylcysteine-*S*-yl)-α-methyldopamine [5-(NAC)-α-MeDA], and 5-(NAC)-N-Me-α-MeDA, as a mixture, at in vivo relevant concentrations (10 μM each compound), and its effects on neuronal mitochondrial trafficking were appraised [8]. Using this experimental design, a reduction was found in overall mitochondrial motility along axonal processes of cultured hippocampal neurons, following mixture exposure for 24 h. This effect was also shown to rely on mitochondrial fusion/fission-dependent mechanisms, as ascertained by the recovery of the mixture's mitochondrial trafficking phenotype in Mfn2 or Drp1 K38A overexpressing neurons [8]. These results also suggested that the mixture of MDMA and its metabolites may target Mfn2 functions, thus resulting in uncontrolled fission and consequent mitochondrial transport impairment.

It seems self-evident that whether mitochondria are not delivered to the right place, at the right time, the neuronal viability will be negatively affected. As such, a

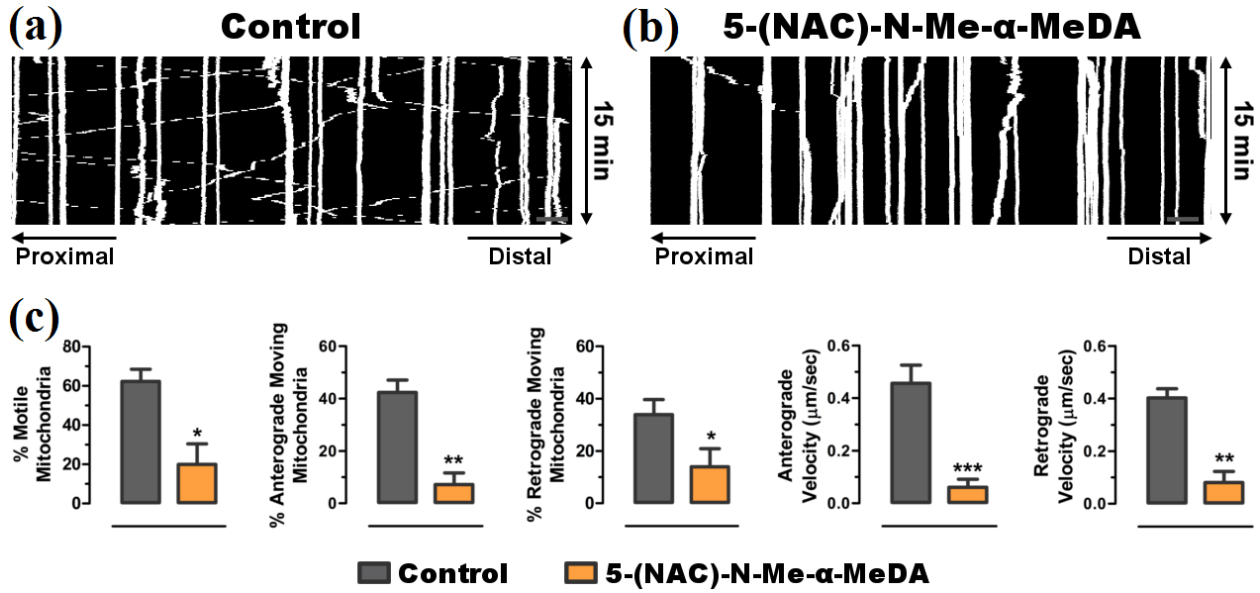

Figure 3: Mitochondrial neuronal trafficking impairment triggered by the MDMA's metabolite 5-(NAC)-N-Me-α-MeDA. (a), (b) Representative kymographs of mitochondrial movement in a single axon under control conditions (a) or after 90 min of exposure to 400 μM of the MDMA's metabolite 5-(NAC)-N-Me-α-MeDA (b). (c) Graphical representation of the percentage of motile mitochondria and velocities, measured in kymographs obtained before and after 5-(NAC)-N-Me-α-MeDA exposure for 90 min. Data represent mean $\pm$SEM of 8 different axons (neurons), from 6 independent experiments ($^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ 5-(NAC)-N-Me-α-MeDA vs. control, Mann-Whitney's test). Scale bar: 10 μm.

close relationship may be proposed between neurotoxic events and mitochondrial trafficking disruptions. It is also clear that disrupted mitochondrial translocation may readily be secondary to other pathogenic events. For example, disruptions on mitochondrial bioenergetics' status, as a primary pathogenic event, are likely to arrest mitochondrial movement, as a consequence of an energetic failure. Nevertheless, it is difficult to attribute a causal association of impaired neuronal mitochondrial trafficking to neuronal damage, though it seems reasonable to consider that they may be closely associated. Neuronal injury mediated by many drugs of abuse, including amphetamine-like drugs [14, 35,47], ethanol [19], cocaine [21] or heroin [22], have been closely associated with bioenergetics' dysfunction. Despite this, the relative contribution of mitochondrial trafficking alterations to this effect remains to be characterized, since it is difficult to attribute any neuronal injury caused by drugs of abuse to compromised mitochondrial movement as a primary cause. At this level, approaches making possible a selective interruption of mitochondrial trafficking, maintaining functional other transport processes, may help in understanding the role of mitochondrial movement dysfunction in the neuronal injury caused by drugs of abuse. In conclusion, as amphetamine-like drugs, such as "ecstasy," promote mitochondrial trafficking impairments, the importance of this effect to their neuropharmacological actions and neurotoxic events deserve further study.

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