NMDA-Induced Ca^{2+} Influx and Mitochondrial ROS Generation Following Cardiac Arrest

McKenna Ann Purkey

Temple University

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Abstract:

Cardiac arrest is associated with a large portion of reperfusion injury to the body (Carr et al., 2008). While this injury is driven in part by oxygen deprivation, it is also mediated by downstream neurotoxic signaling, making it a complex biochemical pathway that many individuals may not fully understand. The major aim is to elucidate the current knowledge behind neurotoxic signaling. This includes identifying relationships between mitochondrial calcium (mitCa^{2+}) homeostasis and dysfunction of the electron transport chain (ETC) (Jaña et al., 2019; Mario et al., 2023). Furthermore, these relationships will demonstrate the importance of N-methyl-D-aspartate (e.g. NMDA) receptor-mediated Ca^{2+} influx as the central driver of post-cardiac injury (Vizi et al., 2013). Within this paper, we will examine the core mechanistic links driven by sustained NMDA activation with a primary focus on Ca^{2+} influx and how this flux disrupts cell balance, leading to the generation of reactive oxygen species (ROS) (Chenna et al., 2022; Chouchani et al., 2014; Murphy & Liu, 2022). NMDA receptor-mediated calcium influx disrupts an overall balance of intracellular mechanisms, particularly mitochondrial calcium homeostasis that is interconnected to electron transport chain disruption (Jaña et al., 2019; Wang et al., 2021). Together, these core processes establish a framework for understanding how upstream cytotoxic developments render oxidative stress. This may also help to identify key regulatory points within this pathway that could give insight into potential therapeutic targets for mitigating post-cardiac injury.

Introduction:

Globally, cardiac arrest remains a leading cause of mortality rates and is associated with significant utilization of hospital resources (Carr et al., 2008; Hamzah et al., 2021). However,

with technological advancements in resuscitation efforts, only modest improvements in survival rates have been observed with outcomes involving the return of spontaneous circulation of blood flow (Carr et al., 2008). Yet, even with successful resuscitation, detrimental effects to the stability of neuronal cells and other neurological consequences are accompanied by restoration. What many have failed to advance is the phenomenon that revolves around post-cardiac injury, not only in the initial state of hypoxic conditions but also to the largely participating biochemical process that is triggered by the primary hypoxic-ischemic insult (Neves et al., 2023). Restoration of blood flow and oxygen supply is critical for survival, yet it fails to prevent the downstream biomolecular processes that ultimately induce neurological cell death (Verma et al., 2022).

One of the earliest events that occurs in these critical moments is an abundance of excess glutamate, an excitatory neurotransmitter, in the extracellular matrix which leads to a sustained activation of ionotropic glutamate receptors on neuronal cells (Brogi et al., 2019; Choi, 1988; Neves et al., 2023). In particular, NMDA receptors play a crucial role in mediating excitotoxic processes towards neurological damage (Hardingham & Bading, 2010; Vizi et al., 2013). When continuous activation of NMDA receptors transpires, it allows for a significant influx of Ca^{2+} ions into the cells, which further disturbs the homeostasis of intracellular calcium (Hansen et al., 2017; Chou et al., 2024). Moreover, subsequent elevation of cytosolic calcium, [Ca^{2+}]cyt, is undertaken by the mitochondria through a supercomplex known formerly as the mitochondrial calcium uniporter (MCU) (Kirichok et al., 2004; Dong et al., 2017). It is widely known that the mitochondria serve a key role in the regulation of energy production, however, the dysregulation of mitCa^{2+} within the organelle is a major repercussion that connects to downstream impairments of the ETC and escalation of reactive oxygen species (Jaña et al., 2019; Murphy & Liu, 2022).

Despite well-established knowledge and understanding of glutamate excitotoxicity and mitochondrial dysfunction, clinical approaches used to assist those under cardiac arrest are primarily focused on the restoration of oxygen delivery and re-circulation (Carr et al., 2008; Hamzah et al., 2021). What fails to be recognized is the underlying biochemical mechanisms that drive neurological damage from the start. Sustained NMDA receptor activation and calcium influx are the central mechanistic drivers linking mitochondrial oxidative metabolism to hypoxia, yet research growth and clinical efforts remain underrepresented (Choi, 1988; Verma et al., 2022). Furthermore, the extent to which NMDA-mediated calcium influx drives mitochondrial uptake and oxidative stress following revascularization, remains incompletely integrated into today's models for post-cardiac arrest (Choi, 1988; Verma et al., 2022). These limitations further highlight the need for targeted therapeutic strategies that are capable of restricting calcium influx in NMDA activation without resulting in failed functionality of the receptor itself.

Collectively, the processes presented within this paper suggest that post-cardiac injury is not only governed by reperfusion, but also by sustained intracellular excitotoxic signaling and mitochondrial dysfunction (Verma et al., 2022; Murphy & Liu, 2022). This stresses the importance of addressing these signaling pathways together with restoring systemic blood flow in order to improve the overall outcomes following cardiac arrest.

NMDA Structure and Activation:

Perpetual availability of extracellular glutamate is mediated by the NMDA receptor, a heterotetrameric ionotropic glutamate receptor, following the loss of excitatory amino acid transporters (EAATs) (Hansen et al., 2017; Vieira et al., 2020). Under these conditions, two molecules of glutamate bind to the GluN2 subunits while a co-agonist, either glycine or D-serine,

binds to the GluN1 subunits respectively in order to facilitate receptor activation and downstream signaling (Chou et al., 2024; Vieira et al., 2020). Both ligands must bind simultaneously to promote a conformational change for activation, this specific conformation induces a domain closure of the Bi-lobed ligand-binding domain (LBD) (Chou et al., 2024). Before the glutamate-gated ion channel opens, activation of AMPA receptors, another type of an ionotropic glutamate receptor, maintains depolarization of the postsynaptic membrane of the neuron, relieving the voltage-dependent block by extracellular magnesium ions (Mg^{2+}) (Choi, 1988; Hansen et al., 2017). The NMDA receptor subsequently opens its transmembrane ion channel allowing an uncontrolled influx of Calcium ions (Ca^{2+}) (Hansen et al., 2017).

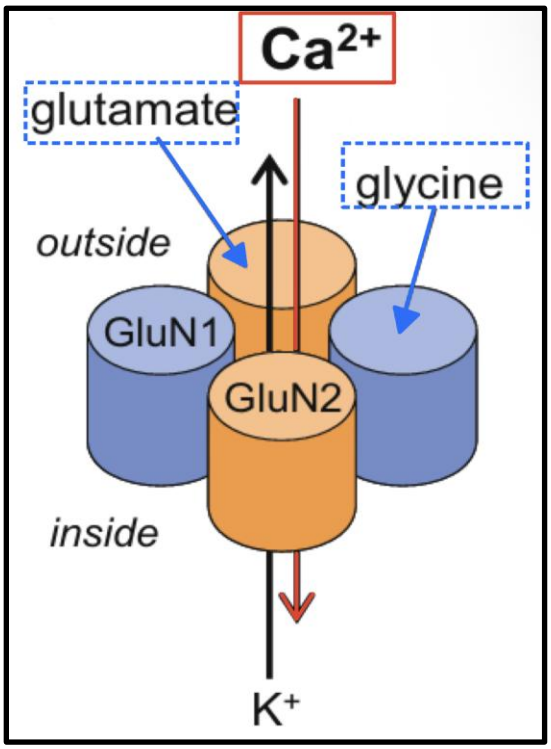

Figure 1: Dual Ligand Requirement of NMDA Receptor. *What is ordinarily a tight regulator for calcium ion entry, the excess of glutamate neurotransmitters deregulates the activation of the NMDA receptor allowing for Ca^{2+} ions (Red solid box) to permeate through the ligand-gated ion channel which overloads the neuron. The GluN1 and GluN2 subunits require glutamate and glycine (or D-serine) to bind to their corresponding subunits for the conformational change and activation to occur (blue dashed boxes). This makes the NMDA receptor the core contributing source of toxic Ca^{2+} influx that initiates downstream mitochondrial strain*

and ultimate neurological destruction that aligns with the importance of this biomolecular mechanism, adapted from reference 16 (Hansen et al., 2017).

Importance of Glutamate Accumulation and Neurotoxicity:

As Choi (1988) has noted, the questions that arise from glutamate-induced neurotoxic injury are in the context of data which suggest the NMDA receptor is important to the mediation of those neuronal damages. The neurotoxic glutamate mechanism primarily aligns with several forms of acute central nervous system (CNS) injury, which include that of hypoxia (Neves et al., 2023). Nevertheless, experimental evidence performed in vitro proposes that blocking the receptor and administering antagonists to the glutamate neurotransmitter can ultimately protect against neuronal injuries, which emphasizes the importance in glutamates role within the mechanistic process (Guo et al., 2017).

Potential of NMDA Receptor Antagonists:

NMDA receptors serve as a prominent target for neuroprotective agents with their central role in calcium mediation (Egunlusi and Joubert, 2024; Guo et al., 2017). Modulating receptor activity through competitive and non-competitive antagonists as well as allosteric modulators based on binding site affinities has been utilized in numerous studies to explore potential agents (Egunlusi and Joubert, 2024; Vizi et al., 2013). However, NMDA receptor activity heavily influences calcium signaling in downstream organelle function. Primarily within mitochondrial function that controls energy production and more importantly, synaptic transmission (Hardingham & Bading, 2010; Murphy and Liu, 2022; Nguyen et al., 2025). This limits clinical success because it may further disrupt mitochondrial function, leading to further impairments of

normal calcium signaling pathways (Murphy and Liu, 2022; Verma et al., 2022). Dysfunction in this case further prevents production of energy and cell signaling needs. This emphasizes the need for site-specific antagonists that can successfully mitigate and control excitatory neurotransmitters such as glutamate without compromising mitochondrial calcium homeostasis.

To better understand the therapeutic potential that revolves around antagonists of the NMDA receptor, experimental techniques are able to directly assess signaling of intracellular calcium. One technique of particular relevance is the use of Fura-2/AM fluorescence imaging of neuronal cultures, where intracellular Ca^{2+} overload can be monitored in real-time following NMDA receptor activation. This method enables evaluation of how NMDA receptor antagonists modulate excitotoxic calcium entry by directly comparing control and NMDA receptor-antagonist conditions. Using this approach, induced calcium influx can also be quantified under such controlled experimental conditions. Additionally, researchers are able to effectively evaluate the performance of such NMDA receptor antagonists in their role of reducing calcium overload. A functional bridge can be established between the effects of mitochondrial pathology and stress pathways to ionotropic receptor dynamics (Calvo et al., 2014).

Ca^{2+} Mitochondrial Uptake: Consequences following Calcium Flux:

Following NMDA receptor mediated calcium influx, a rise in the local concentration of cytosolic Ca^{2+} occurs within the cytoplasm of the neuron, reaching micromolar levels (Kirichok et al., 2004). Mediated permeation of Ca^{2+} through the MCU complex is vital and requires a WDIMEP motif (Asp260 and Glu263), which is a short-conserved sequence of amino acids that help to coordinate calcium movement (Mario et al., 2023). This motif lies within a conserved loop of two transmembrane domains (TM1 and TM2) that are separated by a connector region

that faces outward into intermembrane space (Mario et al., 2023). The MCU complex, an oligomeric inner mitochondrial membrane protein, is a low-affinity uptake system that is crucial for calcium activity (Alevriadou et al., 2021). When cytCa^{2+} rises, the Ca^{2+} ions move freely through an unregulated voltage-dependent anion channel (VDAC) and into intermembrane space (IMS) before crossing the inner mitochondrial membrane (IMM) where the MCU complex resides (Mario et al., 2023; Mustaly-Kalimi et al., 2025).

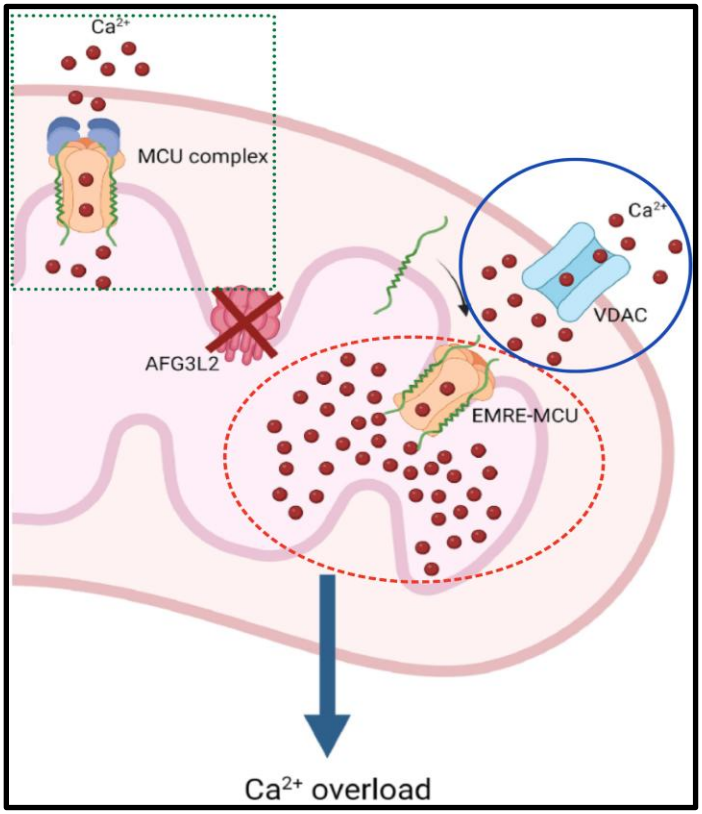

Figure 2: Dysregulation and Mitochondrial Overload with Ca^{2+} of Various Complexes. *Within the VDAC complex (blue circle), cytosolic calcium ions enter via this anion channel and into intermembrane space where those ions are transferred across the IMM to the MCU complex. The calcium ions flow through the pore of the MCU to reach the mitochondrial matrix. Dysregulation of elevated calcium ion flow occurs via the MCU complex (green dotted box) and EMRE-MCU complex (red dashed oval) when regulators EMRE (Essential MCU Regulator), MICU, and MICU2 essentially become less effective, further preventing the channel into the matrix from being closed. The EMRE-MCU complex shown exhibits a constitutively active state reflecting an influx and overload of Ca^{2+}*

ions within the matrix driven by mitochondrial membrane potential (ΔΨm), adapted from reference 20 (Mario et al., 2023).

Before elevated mitochondrial Ca^{2+} ions (mitCa^{2+}) enter, a supercomplex appears in dimeric form and includes the regulators MCU, EMRE, MICU1, and MICU2. The MCU must construct an inner tetrameric membrane channel; in doing so, the transmembrane domain (TMD), responsible for conductance of the calcium ions, assembles a pore entrance containing E264 residues that form a selectivity filter for Ca^{2+} permeation. A coiled-coiled domain (CCD) of the MCU is connected to the TMD which stabilizes conformational changes of the MCU. EF-hands, consisting of two alpha helices connected by a small loop residue, are the binding domains for Ca^{2+} where MICU1 acts as the cooperative activator with MICU2 by forming obligate heterodimers which induces a conformational change, further unblocking the pores entrance and binding to activated Ca^{2+}. The interaction between these two dimers attenuates EMRE-dependent regulation of the MCU complex all within the inner mitochondrial membrane (Mario et al., 2023).

Upon this occurrence, mitochondrial membrane potential (ΔΨm) drives Ca^{2+} ions into the mitochondrial matrix. This potential is generated by the electron transport chain (ETC) as an electrochemical gradient, which allows import and export of ions as well as differing proteins and other molecules such as metabolites (Jaña et al., 2019). This influx of Ca^{2+} ions overstimulates the gatekeeping function of regulating proteins, MICU1 and MICU2, to which stabilizes an open and active conformation of the pore causing the overload of Ca^{2+} into the matrix (Mario et al., 2023).

Thus, uptake of Ca^{2+} into the mitochondria matrix via the MCU supercomplex serves as a pivotal biomolecular bridge through which NMDA-mediated calcium influx transitions into irreversible mitochondrial stress and dysfunction (Verma et al., 2022; Murphy and Liu, 2022). Accordingly, MCU-mediated Ca^{2+} influx is not an isolated process that works independently in neurological destruction following cardiac arrest and hypoxia (Verma et al., 2022; Wang et al., 2021). The mechanism produces physiological consequences that exceed capacity of the organelle, further elucidating the context to which the TCA cycle is altered following uncontrollable metabolic balance (Chenna et al., 2022; Wang et al., 2021).

Dysfunction of the Electron Transport Chain & Generation of ROS Production:

Ca^{2+} uptake alters energy production rates that are traditionally mediated by the mitochondrial calcium uniporter) (Jaña et al., 2019; Donato D'Angelo and Rizzuto, 2023). Prolonged disruption of the electron transport chain due to excessive $\Delta\Psi m$ hyperpolarization links to mitochondrial ROS production (Chenna et al., 2022). Elevated mitochondrial Ca^{2+} amplifies the activity of key enzymes within the tricarboxylic acid (TCA) cycle that are known as pyruvate dehydrogenase, isocitrate dehydrogenase, and α-Ketoglutarate dehydrogenase (Chenna et al., 2022; Murphy and Liu, 2022). This activity generates excess levels of nicotinamide adenine dinucleotides (NADH) that exceed oxidative capacity of the mitochondrial respiratory chain (Chenna et al., 2022).

Under normal physiological conditions, the coenzyme NADH serves as the primary electron donor to Complex I of the electron transport chain (ETC), which supports regulated electron flow for ATP production. These electrons are transferred through flavin mononucleotide (FMN) to reduce ubiquinone (Q), which ultimately forms ubiquinol (QH_2). Under these

favorable conditions, electron transfer between these molecules contributes highly to the translocation of four H^+ protons from the mitochondrial matrix into IMS for the proton motive force that drives primary ATP synthesis (Chenna et al., 2022).

However, due to overload of Ca^{2+} stimulating TCA cycle flux and NADH production, Complex I of the ETC receives a greater supply of electrons which overwhelms the downstream Complexes (III & IV) that are not able to increase flow proportionally (Chenna et al., 2022). Consequently, upstream complexes and associated carriers, such as ubiquinone and FMN, of the ETC are forced into a highly reduced state (Chenna et al., 2022). Upon reperfusion when oxygen is re-introduced to cells, a series of interconnected metabolic changes occur; including the rapid oxidation of succinate dehydrogenase via Complex II, driving a large flow of electrons directly into the ubiquinone (Q) pool and excessively increases levels of QH_2 (Chenna et al., 2022; Chouchani et al., 2014).

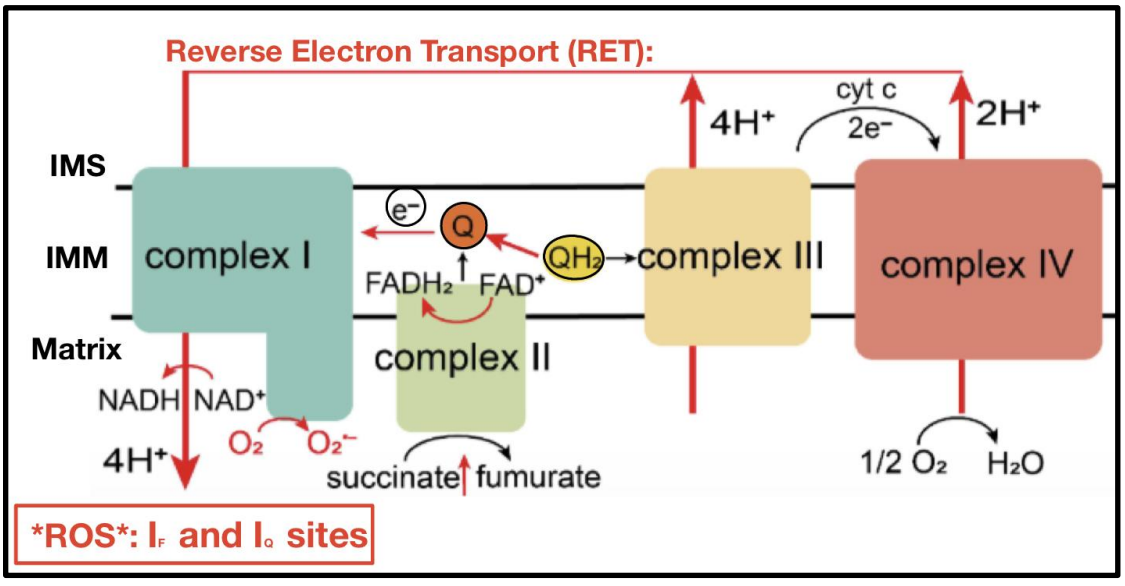

Figure 3: Calcium (Ca^{2+}) Driven Mitochondrial Reverse Electron Transport (RET). *The increase in NADH levels and hyperpolarized $\Delta\Psi m$ induces a drive in electron (Black circle) flow into a reverse order that reverts QH_2 (Highlighted black circle) back into Complex I.*

Electrons are forced into an extremely reduced state where superoxide is generated and accumulates at the I_F and I_Q sites that release substantially increased levels of reactive oxygen species (Bold red box). Once in the mitochondrial matrix, they promote mitochondrial dysfunction, adapted from reference 3 (Bao et al., 2025).

Such conditions that promote reverse electron transport (RET) arise upon re-establishment of mitochondrial membrane potential, resulting in a hyperpolarized state as oxygen is rapidly reintroduced, thereby restoring proton pumping (Chouchani et al., 2014). Together this creates thermodynamically unfavorable conditions that promote the reverse flow of electrons in the electron transport chain (Chenna et al., 2022; Chouchani et al., 2014). The electrons begin to flow in reverse from QH_2 back into Complex I forcing electrons into a highly reduced state where they primarily accumulate at the I_F and I_Q sites, generating a reduction (gain of electrons) to molecular oxygen which ultimately generates superoxide (Chenna et al., 2022; Chouchani et al., 2014. In parallel, NAD^+ continues to rapidly revert back to NADH, shifting the $NADH/NAD^+$ ratio, where ROS generation further enhances at the I_F and I_Q sites (Chenna et al., 2022).

Notably, RET generates a significantly higher production of ROS than that of forward ETC (FET). This production is highly dependent on $NADH/NAD^+$ and QH_2/Q ratios as well as small-scale increases in the $\Delta\Psi m$. A key difference that sets RET apart from FET is that the mitochondria is able to efficiently remove the generated ROS during FET as a result of scavenging mechanisms, such as enzymatic antioxidants (Chenna et al., 2022).

The RET mechanism translates back to NMDA overactivation with its substantially high calcium influx that unites all biochemical processes into a single pathway. The findings position

RET as the critical consequence that leads to automatic dysfunction in neuronal cells (Verma et al., 2022). The direct correlation between oxidative stress in the mitochondria and initial neurotoxicity produced by glutamate neurotransmitters highlights that disruption in NMDA activity extends beyond synaptic function, as it produces additional results in numerous defects at the cellular level (Verma et al., 2022). In the context of cardiac arrest, the reperfusion that stems from this sudden medical condition and the rapid reintroduction of oxygen is what strongly promotes RET-driven ROS generation. This is what uniquely distinguishes this specific pathway from other sources of ROS generation (Chouchani et al., 2014).

Therapeutic Strategies Targeting NMDA-Mediated Ca^{2+} Neurotoxicity:

Recent **s**tudies have demonstrated pharmacological intervention with the use of competitive ligand-binding site antagonists of the NMDA receptor. Glycine-site competitive antagonist (MDL 105,519) and glutamate-site competitive antagonist (CGP 070667) have shown efficiency in blocking ligands, glutamate and glycine, from binding to their respective NMDA receptor subunits. The study exhibited competitive inhibition of receptor activation and successful reduction of neurotoxic Ca^{2+} overload in non-neuronal (human embryonic kidney-derived) cells. These site-specific antagonists also demonstrated a dose-dependent activation in a controlled environment while using exogenous ligands, allowing for accurate determination of EC_{50} values. For context, EC_{50} values represent the concentration of an antagonist required to produce half-maximal response in inhibition to receptor activity; These values provide a quantitative measure of antagonist potency and can be applied to the optimization of dosing strategies. In relation to ligand-binding site antagonists, EC_{50} determinations provide a means for comparing both glycine and glutamate-site inhibition efficiency in suppressing receptor

activation. The study's findings provided additional information on the characterization of the NMDA heterotetrametric structure, as well as the contributions made to Ca^{2+} permeability by specific subunit composition (Guo et al., 2017).

However, a range of limitations to the study include variability in NMDA receptor subtype expression based on differing cellular systems, while also using experimentally simplified in vitro models. This model is suggested to not fully replicate in vivo excitotoxic conditions that are typically observed in post-cardiac injury. Additionally, the differential efficiency of both ligand-binding site antagonists implies that single-site inhibition alone may not achieve full suppression of pathological calcium influx. Future directions in this therapeutic approach may therefore benefit from multi-targeted strategies for NMDA receptor modulation along with further regulation of downstream mitochondrial calcium accumulation. Furthermore, the application of more physiologically represented conditions that better replicate hypoxic-ischemic dynamics would strengthen translational relevance and experimental findings (Guo et al., 2017).

Conclusion:

Unifying the various mechanistic processes underlying neurological injury following cardiac arrest reveals a sequence of interdependent calcium-mediated events. Beginning with sustained NMDA activation, the cascade progresses following the influx of Ca^{2+} ions as they travel into the mitochondrial compartment (Hardingham and Bading, 2010). The pathological influx of those Ca^{2+} ions disrupts the overall homeostasis of those ions within numerous intracellular mechanisms (Verma et al., 2022). When cytosolic Ca^{2+} overload reaches the mitochondrial matrix via the MCU complex, it induces an irreversible imbalance that triggers

complete metabolic dysfunction of the cell's functioning system (Verma et al., 2022; Murphy and Liu, 2022). Under conditions of calcium overload, mitochondrial membrane potential ($\Delta\Psi m$) creates an electrochemical gradient across the inner mitochondrial membrane (Chenna et al., 2022; Jaña et al., 2019). The MCU complex becomes overstimulated by this gradient, promoting the stabilization of open-channel conformation that not only drives Ca^{2+} ions inwards but also overwhelms the ETC and fundamentally propagates dysfunction and electron leakage through RET (Balderas et al., 2022; Chenna et al., 2022; Murphy and Liu, 2022). Collectively, these processes that occur during reperfusion, amplify the generation of reactive oxygen species that lead to oxidative stress (Chouchani et al., 2014; Murphy and Liu, 2022; Wang et al., 2021).

This entire biomolecular cascade positions NMDA receptor-mediated Ca^{2+} influx as the primary and leading mechanistic link between hypoxia (following cardiac arrest) and indefinite neuronal and cardiomyocyte damage resulting in death (Hardingham and Bading, 2010; Verma et al., 2022). In doing so, organizing these processes into a unified mechanistic pathway gives a more comprehensive approach to understanding post-cardiac events that have otherwise appeared as disconnected pathophysiology (Neves et al., 2023). While the framework presented in this paper improves conceptual integration of post-cardiac pathology, there are many mechanistic gaps that remain unclear.

Although there has been substantial progress in understanding the characterization of this pathway, there are critical questions that still remain unanswered. While NMDA receptors play a critical role in neurodegenerative conditions such as this, their specific subtypes are unique in a way that remains to be fully understood (Vieira et al., 2020; Egunlusi and Joubert, 2024). This gives rise to additional questions regarding physiological calcium thresholds within the mitochondria that are not fully defined, particularly the extent to which point in calcium influx,

under dynamically changing conditions such as reperfusion, drives irreversible neurological damage (Murphy and Liu, 2022; Wang et al., 2021)? Various experimental models show extensive variability in their setups which include differences in cell types, reoxygenation methods, mitochondrial dysfunction, and combined hypoxic-ischemic stress, which set limitations to effective treatment strategies in such a scenario (Guo et al., 2017; Neves et al., 2023).

Current research and clinical studies have yet to sufficiently bridge the critical gap between stabilization after reperfusion and deep intracellular biochemical mechanisms that prevent full physiological recovery (Carr et al., 2008; Murphy and Liu, 2022). Drawing from observations made, NMDA receptors present the greatest potential in future research through the mitigation of neurotoxic events and regulation of associated mitochondrial calcium fluctuation in neuronal and cardiomyocyte cells (Egunlusi and Joubert, 2024). This direction in research can be supported by calcium-imaging techniques, where Fura-2/AM-based cellular assays can allow for a direct assessment of NMDA receptor function and Ca^{2+}dynamics (Calvo et al., 2014). By targeting NMDA activity through the receptor's specific subtypes and subunits, further experimental approaches could potentially preserve physiological signaling while ultimately reducing related mitochondrial pathways and oxidative stress (Guo et al., 2017). This, in turn, could provide insight into how they contribute to the overall phenomenon of post-cardiac and hypoxic injury. Similarly, providing the means to prevent overload through the MCU, and selectively regulate mitCa^{2+} into the matrix without disruption of metabolic function, can ultimately lead to refined neuroprotective agents following cardiac arrest.

AI tools were not used to generate scientific content. All scientific content, analysis, and conclusions were reviewed and revised by the author.

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