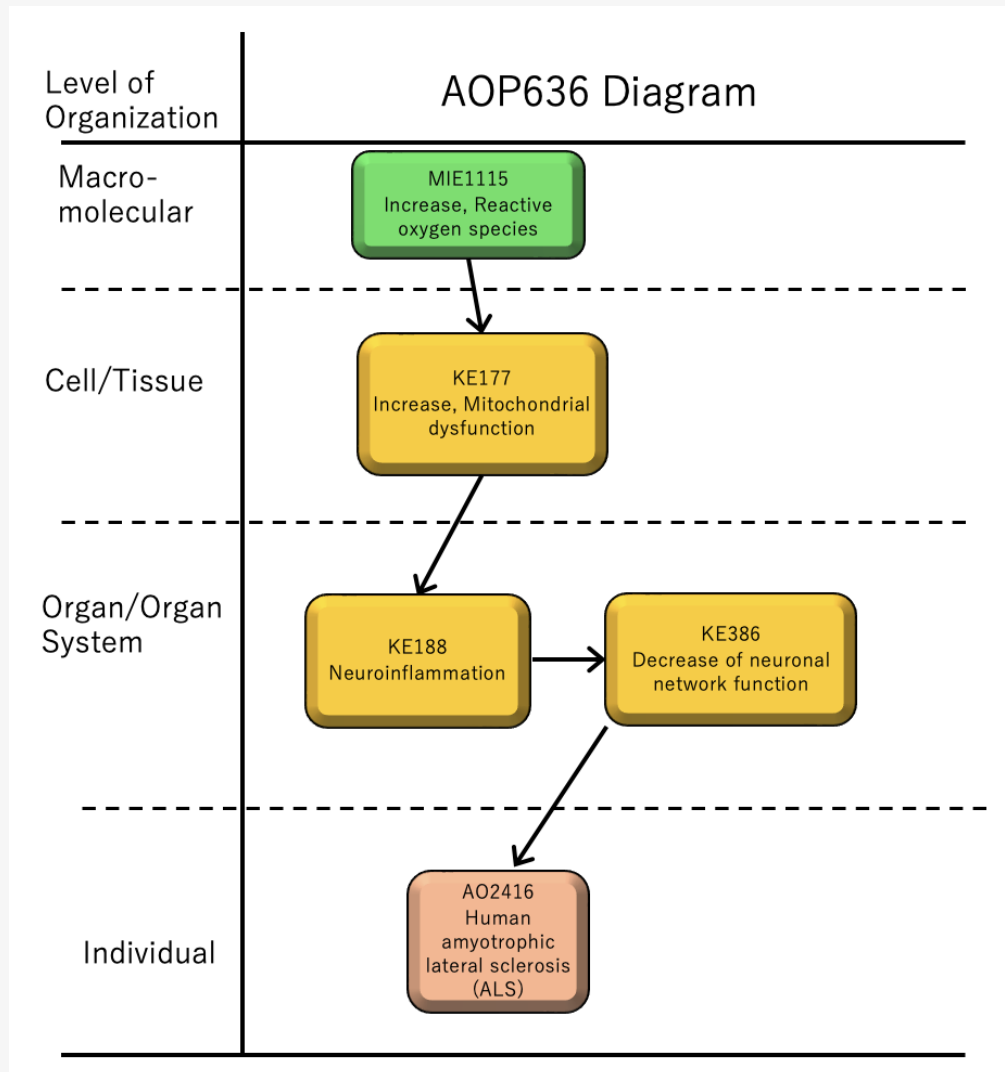


AOP ID and Title:

AOP 636: Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)

Short Title: Increase in ROS leading to human ALS

Graphical Representation**Authors**

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Author status**OECD status OECD project SAAOP status**

Under development: Not open for comment. Do not cite

Coaches

You
Song

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the impairment of motor neurons in the brain and spinal cord, leading to skeletal muscle atrophy and eventual death due to respiratory muscle paralysis. Currently, fundamental curative treatments remain unestablished. The objective of this study is to develop an Adverse Outcome Pathway (AOP) for ALS to elucidate its underlying pathological mechanisms. By analyzing molecular networks using RNA-seq data and synthesizing existing literature, we have proposed a draft AOP. This pathway delineates the progression from an initial rise in reactive oxygen species (ROS) and oxidative stress to neurodegeneration and motor neuron death, mediated by key events such as TDP-43 aggregation, mitochondrial dysfunction, neuroinflammation, and axonal transport failure. In developing a quantitative AOP (qAOP), it is essential to assess the quantifiability of each Key Event (KE) and Key Event Relationship (KER) to accurately model the network's functional relationships. Current AOP636 starts with MIE1115, an increase in ROS, links to KE177, an increase in mitochondrial dysfunction, KE188, neuroinflammation, and KE386, a decrease in neuronal network function, and leads to AO2416 human ALS. We need to develop a new KE2416 on ALS and KERs with a quantitative understanding.

Summary of the AOP**Events****Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)**

Sequence	Type	Event ID	Title	Short name
	MIE	1115	Increase, Reactive oxygen species	Increase, ROS
	KE	177	Increase, Mitochondrial dysfunction	Increase, Mitochondrial dysfunction
	KE	188	Neuroinflammation	Neuroinflammation
	KE	386	Decrease of neuronal network function	Neuronal network function, Decreased
	AO	2416	human amyotrophic lateral sclerosis (ALS)	ALS

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increase, Reactive oxygen species	adjacent	Increase, Mitochondrial dysfunction	High	
Increase, Mitochondrial dysfunction	adjacent	Neuroinflammation	High	
Neuroinflammation	adjacent	Decrease of neuronal network function	High	
Decrease of neuronal network function	adjacent	human amyotrophic lateral sclerosis (ALS)	High	

Stressors

Name	Evidence
Reactive oxygen species	

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage Evidence

Adult High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Sex Applicability

Sex Evidence

Male High

Female High

References

Appendix 1

List of MIEs in this AOP

Event: 1115: Increase, Reactive oxygen species

Short Name: Increase, ROS

Event Component

Process	Object	Action
reactive oxygen species biosynthetic process	reactive oxygen species	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:186 - unknown MIE leading to renal failure and mortality	KeyEvent
Aop:213 - Inhibition of fatty acid beta oxidation leading to nonalcoholic steatohepatitis (NASH)	KeyEvent
Aop:303 - Frustrated phagocytosis-induced lung cancer	KeyEvent
Aop:383 - Inhibition of Angiotensin-converting enzyme 2 leading to liver fibrosis	KeyEvent
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:384 - Hyperactivation of ACE/Ang-II/AT1R axis leading to chronic kidney disease	KeyEvent
Aop:396 - Deposition of ionizing energy leads to population decline via impaired meiosis	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	KeyEvent
Aop:416 - Aryl hydrocarbon receptor activation leading to lung cancer through IL-6 toxicity pathway	KeyEvent
Aop:418 - Aryl hydrocarbon receptor activation leading to impaired lung function through AHR-ARNT toxicity pathway	KeyEvent
Aop:386 - Deposition of ionizing energy leading to population decline via inhibition of photosynthesis	KeyEvent

AOP ID and Name	Event Type
Aop:387 - Deposition of ionising energy leading to population decline via mitochondrial dysfunction	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	MolecularInitiatingEvent
Aop:492 - Glutathione conjugation leading to reproductive dysfunction via oxidative stress	KeyEvent
Aop:497 - ERα inactivation alters mitochondrial functions and insulin signalling in skeletal muscle and leads to insulin resistance and metabolic syndrome	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:505 - Reactive Oxygen Species (ROS) formation leads to cancer via inflammation pathway	MolecularInitiatingEvent
Aop:513 - Reactive Oxygen (ROS) formation leads to cancer via Peroxisome proliferation-activated receptor (PPAR) pathway	MolecularInitiatingEvent
Aop:521 - Essential element imbalance leads to reproductive failure via oxidative stress	KeyEvent
Aop:540 - Oxidative Stress in the Fish Ovary Leads to Reproductive Impairment via Reduced Vitellogenin Production	MolecularInitiatingEvent
Aop:462 - Activation of reactive oxygen species leading the atherosclerosis	MolecularInitiatingEvent
Aop:299 - Deposition of energy leading to population decline via DNA oxidation and follicular atresia	KeyEvent
Aop:311 - Deposition of energy leading to population decline via DNA oxidation and oocyte apoptosis	KeyEvent
Aop:331 - Reactive oxygen species leading to growth inhibition via lipid peroxidation and cell death	MolecularInitiatingEvent
Aop:327 - Excessive reactive oxygen species production leading to mortality (1)	MolecularInitiatingEvent
Aop:328 - Excessive reactive oxygen species production leading to mortality (2)	MolecularInitiatingEvent
Aop:329 - Excessive reactive oxygen species production leading to mortality (3)	MolecularInitiatingEvent
Aop:330 - Excessive reactive oxygen species production leading to mortality (4)	MolecularInitiatingEvent
Aop:26 - Calcium-mediated neuronal ROS production and energy imbalance	KeyEvent
Aop:534 - Succinate dehydrogenase (SDH) inhibition leads to oxidative stress	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:488 - Increased reactive oxygen species production leading to decreased cognitive function	MolecularInitiatingEvent
Aop:298 - Increase in reactive oxygen species (ROS) leading to human treatment-resistant gastric cancer	MolecularInitiatingEvent
Aop:27 - Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11)	KeyEvent
Aop:511 - The AOP framework on ROS-mediated oxidative stress induced vascular disrupting effects	MolecularInitiatingEvent
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:423 - Toxicological mechanisms of hepatocyte apoptosis through the PARP1 dependent cell death pathway	MolecularInitiatingEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	MolecularInitiatingEvent
Aop:282 - Adverse outcome pathway on photochemical toxicity initiated by light exposure	MolecularInitiatingEvent
Aop:569 - Decreased DNA methylation of FAM50B/PTCHD3 leading to IQ loss of children via PI3K-Akt pathway	KeyEvent
Aop:595 - Emerging OPFRS reproductive outcome pathway	MolecularInitiatingEvent

AOP ID and Name	Event Type
Aop:596 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell injury/death	MolecularInitiatingEvent
Aop:598 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and reduced cell proliferation	MolecularInitiatingEvent
Aop:599 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and cell injury/death	MolecularInitiatingEvent
Aop:600 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell growth	MolecularInitiatingEvent
Aop:601 - Excessive reactive oxygen species leading to growth inhibition via fatty acid oxidation and reduced cell proliferation	MolecularInitiatingEvent
Aop:602 - Excessive reactive oxygen species leading to growth inhibition via oxidative DNA damage	MolecularInitiatingEvent
Aop:603 - Excessive reactive oxygen species leading to growth inhibition via protein oxidation and cell cycle disruption	MolecularInitiatingEvent
Aop:608 - Thyroid Hormone Excess Leading to Reduced, Swimming Performance via Hypomyelination	KeyEvent
Aop:613 - Peroxisome proliferator-activated receptor alpha activation leading to early life stage mortality via increased reactive oxygen species production	KeyEvent
Aop:622 - Calcineurin inhibitor induced nephrotoxicity leading to kidney failure	KeyEvent
Aop:636 - Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	MolecularInitiatingEvent
Aop:638 - Co-exposure to microplastics and cadmium leading to progression from NAFLD to liver tumorigenesis	MolecularInitiatingEvent
Aop:472 - DNA adduct formation leading to kidney failure	KeyEvent
Aop:324 - Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell cycle disruption	MolecularInitiatingEvent
Aop:325 - Reactive oxygen species leading to growth inhibition via oxidative DNA damage and cell death	MolecularInitiatingEvent
Aop:326 - Reactive oxygen species leading to growth inhibition via lipid peroxidation and decreased cell proliferation	MolecularInitiatingEvent
Aop:332 - Reactive oxygen species leading to growth inhibition via protein oxidation and decreased cell proliferation	MolecularInitiatingEvent
Aop:333 - Reactive oxygen species leading to growth inhibition via protein oxidation and cell death	MolecularInitiatingEvent
Aop:647 - ROS formation leading to growth inhibition modulated by the Keap1-Nrf2 antioxidant response	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI
human	Homo sapiens	Moderate	NCBI
human and other cells in culture	human and other cells in culture	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI
crustaceans	Daphnia magna	High	NCBI
Lemna minor	Lemna minor	High	NCBI
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High
Mixed	High

ROS is a normal constituent found in all organisms, *lifestages*, and *sexes*.

Key Event Description

Biological State: increased reactive oxygen species (ROS)

Biological compartment: an entire cell -- may be cytosolic, may also enter organelles.

Reactive oxygen species (ROS) are O_2^- -derived molecules that can be both free radicals (e.g. superoxide, hydroxyl, peroxy, alkoxy) and non-radicals (hypochlorous acid, ozone and singlet oxygen) (Bedard and Krause 2007; Ozcan and Ogun 2015). ROS production occurs naturally in all kinds of tissues inside various cellular compartments, such as mitochondria and peroxisomes (Drew and Leeuwenburgh 2002; Ozcan and Ogun 2015). Furthermore, these molecules have an important function in the regulation of several biological processes – they might act as antimicrobial agents or triggers of animal gamete activation and capacitation (Goud et al. 2008; Parrish 2010; Bisht et al. 2017).

However, in environmental stress situations (exposure to radiation, chemicals, high temperatures) these molecules have its levels drastically increased, and overly interact with macromolecules, namely nucleic acids, proteins, carbohydrates and lipids, causing cell and tissue damage (Brieger et al. 2012; Ozcan and Ogun 2015).

Reactive oxygen species (ROS) refers to the chemical species superoxide, hydrogen peroxide, and their secondary reactive products. In the biological context, ROS are signaling molecules with important roles in cell energy metabolism, cell proliferation, and fate. Therefore, balancing ROS levels at the cellular and tissue level is an important part of many biological processes. Disbalance, mainly an increase in ROS levels, can cause cell dysfunction and irreversible cell damage.

ROS are produced from both exogenous stressors and normal endogenous cellular processes, such as the mitochondrial electron transport chain (ETC). Inhibition of the ETC can result in the accumulation of ROS. Exposure to chemicals, heavy metal ions, or ionizing radiation can also result in increased production of ROS. Chemicals and heavy metal ions can deplete cellular antioxidants reducing the cell's ability to control cellular ROS and resulting in the accumulation of ROS. Cellular antioxidants include glutathione (GSH), protein sulfhydryl groups, superoxide dismutase (SOD).

ROS are radicals, ions, or molecules that have a single unpaired electron in their outermost shell of electrons, which can be categorized into two groups: free oxygen radicals and non-radical ROS [Liou et al., 2010].

<Free oxygen radicals>

superoxide	$O_2^{\cdot-}$
hydroxyl radical	$\cdot OH$
nitric oxide	$NO^{\cdot}$
organic radicals	$R^{\cdot}$
peroxyl radicals	$ROO^{\cdot}$

alkoxyl radicals	RO·
thiyl radicals	RS·
sulfonyl radicals	ROS·
thiyl peroxy radicals	RSOO·
disulfides	RSSR

<Non-radical ROS>

hydrogen peroxide	H ₂ O ₂
singlet oxygen	¹ O ₂
ozone/trioxygen	O ₃
organic hydroperoxides	ROOH
hypochlorite	ClO ⁻
peroxynitrite	ONOO ⁻
nitrosoperoxycarbonate anion	O=NOOCO ₂ ⁻
nitrocarbonate anion	O ₂ NOCO ₂ ⁻
dinitrogen dioxide	N ₂ O ₂
nitronium	NO ₂ ⁺
highly reactive lipid- or carbohydrate-derived carbonyl compounds	

Potential sources of ROS include NADPH oxidase, xanthine oxidase, mitochondria, nitric oxide synthase, cytochrome P450, lipoxygenase/cyclooxygenase, and monoamine oxidase [Granger et al., 2015]. ROS are generated through NADPH oxidases consisting of p47^{phox} and p67^{phox}. ROS are generated through xanthine oxidase activation in sepsis [Ramos et al., 2018]. Arsenic produces ROS [Zhang et al., 2011]. Mitochondria-targeted paraquat and metformin mediate ROS production [Chowdhury et al., 2020]. ROS are generated by bleomycin [Lu et al., 2010]. Radiation induces dose-dependent ROS production [Ji et al., 2019].

ROS are generated in the course of cellular respiration, metabolism, cell signaling, and inflammation [Dickinson and Chang 2011; Egea et al. 2017]. Hydrogen peroxide is also made by the endoplasmic reticulum in the course of protein folding. Nitric oxide (NO) is produced at the highest levels by nitric oxide synthase in endothelial cells and phagocytes. NO production is one of the main mechanisms by which phagocytes kill bacteria [Wang et al., 2017]. The other species are produced by reactions with superoxide or peroxide, or by other free radicals or enzymes.

ROS activity is principally local. Most ROS have short half-lives, ranging from nano- to milliseconds, so diffusion is limited, while reactive nitrogen species (RNS) nitric oxide or peroxynitrite can survive long enough to diffuse across membranes [Calcerrada et al. 2011]. Consequently, local concentrations of ROS are much higher than average cellular concentrations, and signaling is typically controlled by colocalization with redox buffers [Dickinson and Chang 2011; Egea et al. 2017].

Although their existence is limited temporally and spatially, ROS interact with other ROS or with other nearby molecules to produce more ROS and participate in a feedback loop to amplify the ROS signal, which can increase RNS. Both ROS and RNS also move into neighboring cells, and ROS can increase intracellular ROS signaling in neighboring cells [Egea et al. 2017].

In the primary event, photoreactive chemicals are excited by the absorption of photon energy. The energy of the photoactivated chemicals transfer to oxygen and then generates the reactive oxygen species (ROS), including superoxide (O₂⁻) via type I reaction and singlet oxygen (¹O₂) via type II reaction, as principal intermediate species in phototoxic reaction [Foote, 1991, Onoue et al. , 2009].

How it is Measured or Detected

Photocolorimetric assays (Sharma et al. 2017; Griending et al. 2016) or through commercial kits purchased from specialized companies.

Yuan, Yan, et al., (2013) described ROS monitoring by using H₂-DCF-DA, a redox-sensitive fluorescent dye. Briefly, the harvested cells were incubated with H₂-DCF-DA (50 μmol/L final concentration) for 30 min in the dark at 37°C. After treatment, cells were immediately washed twice, re-suspended in PBS, and analyzed on a BD-FACS Aria flow cytometry. ROS generation was based on fluorescent intensity which was recorded by excitation at 504 nm and emission at 529 nm.

Lipid peroxidation (LPO) can be measured as an indicator of oxidative stress damage Yen, Cheng Chien, et al., (2013).

Chattopadhyay, Sukumar, et al. (2002) assayed the generation of free radicals within the cells and their extracellular release in the medium by addition of yellow NBT salt solution (Park et al., 1968). Extracellular release of ROS converted NBT to a purple colored formazan. The cells were incubated with 100 ml of 1 mg/ml NBT solution for 1 h at 37 °C and the product formed was assayed at 550 nm in an Anthos 2001 plate reader. The observations of the 'cell-free system' were confirmed by cytological examination of parallel set of explants stained with chromogenic reactions

for NO and ROS.

On the basis of the pathogenesis of drug-induced phototoxicity, a reactive oxygen species (ROS) assay was proposed to evaluate the phototoxic risk of chemicals. The ROS assay can monitor generation of ROS, such as singlet oxygen and superoxide, from photoirradiated chemicals, and the ROS data can be used to evaluate the photoreactivity of chemicals (Onoue et al. , 2014, Onoue et al. , 2013, Onoue and Tsuda, 2006). The ROS assay is a recommended approach by guidelines to evaluate the phototoxic risk of chemicals (ICH, 2014, PCPC, 2014).

<Direct detection>

Many fluorescent compounds can be used to detect ROS, some of which are specific, and others are less specific.

□ ROS can be detected by fluorescent probes such as *p*-methoxy-phenol derivative [Ashoka et al., 2020].

□ Chemiluminescence analysis can detect the superoxide, where some probes have a wider range for detecting hydroxyl radical, hydrogen peroxide, and peroxynitrite [Fuloria et al., 2021].

□ ROS in the blood can be detected using superparamagnetic iron oxide nanoparticles (SPION)-based biosensor [Lee et al., 2020].

□ Hydrogen peroxide (H₂O₂) can be detected with a colorimetric probe, which reacts with H₂O₂ in a 1:1 stoichiometry to produce a bright pink colored product, followed by the detection with a standard colorimetric microplate reader with a filter in the 540-570 nm range.

□ The levels of ROS can be quantified using multiple-step amperometry using a stainless steel counter electrode and non-leak Ag|AgCl reference node [Flaherty et al., 2017].

□ Singlet oxygen can be measured by monitoring the bleaching of *p*-nitrosodimethylaniline at 440 nm using a spectrophotometer with imidazole as a selective acceptor of singlet oxygen [Onoue et al., 2014].

<Indirect Detection>

Alternative methods involve the detection of redox-dependent changes to cellular constituents such as proteins, DNA, lipids, or glutathione [Dickinson and Chang 2011; Wang et al. 2013; Griending et al. 2016]. However, these methods cannot generally distinguish between the oxidative species behind the changes and cannot provide good resolution for the kinetics of oxidative activity.

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List of Key Events in the AOP

Event: 177: Increase, Mitochondrial dysfunction

Short Name: Increase, Mitochondrial dysfunction

Event Component

Process	Object	Action
	mitochondrion	functional change

AOPs Including This Key Event

AOP ID and Name

Event Type

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent
Aop:77 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1	KeyEvent
Aop:79 - Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure	KeyEvent
Aop:80 - Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure	KeyEvent
Aop:87 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KeyEvent
Aop:3 - Inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:178 - Nicotinic acetylcholine receptor activation contributes to mitochondrial dysfunction and leads to colony loss/failure	KeyEvent
Aop:200 - Estrogen receptor activation leading to breast cancer	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:437 - Inhibition of mitochondrial electron transport chain (ETC) complexes leading to kidney toxicity	KeyEvent
Aop:423 - Toxicological mechanisms of hepatocyte apoptosis through the PARP1 dependent cell death pathway	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to left ventricular function decrease via increased myocardial oxidative stress	KeyEvent
Aop:480 - Mitochondrial complexes inhibition leading to heart failure via decreased ATP production	KeyEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	KeyEvent
Aop:509 - Nrf2 inhibition leading to vascular disrupting effects through activating apoptosis signal pathway and mitochondrial dysfunction	KeyEvent
Aop:511 - The AOP framework on ROS-mediated oxidative stress induced vascular disrupting effects	KeyEvent
Aop:256 - Inhibition of mitochondrial DNA polymerase gamma leading to kidney toxicity	KeyEvent
Aop:258 - Renal protein alkylation leading to kidney toxicity	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:205 - AOP from chemical insult to cell death	KeyEvent
Aop:335 - AOP for urothelial carcinogenesis due to chemical cytotoxicity by mitochondrial impairment	MolecularInitiatingEvent
Aop:362 - Immune mediated hepatitis	KeyEvent
Aop:130 - Phospholipase A2 (LPLA2) inhibitors leading to hepatotoxicity	KeyEvent
Aop:497 - ERα inactivation alters mitochondrial functions and insulin signalling in skeletal muscle and leads to insulin resistance and metabolic syndrome	KeyEvent
Aop:34 - LXR activation leading to hepatic steatosis	KeyEvent

AOP ID and Name	Event Type
Aop:447 - Kidney failure induced by inhibition of mitochondrial electron transfer chain through apoptosis, inflammation and oxidative stress pathways	KeyEvent
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	KeyEvent
Aop:530 - Endocytotic lysosomal uptake leads to intestinal barrier disruption	KeyEvent
Aop:587 - Inhibition of the mitochondrial complex III of nigro-striatal neurons leads to parkinsonian motor deficits	KeyEvent
Aop:588 - Inhibition of the mitochondrial complex II of nigro-striatal neurons leads to parkinsonian motor deficits	KeyEvent
Aop:589 - Inhibition of the mitochondrial complex IV of nigro-striatal neurons leads to parkinsonian motor deficits	KeyEvent
Aop:595 - Emerging OPFRS reproductive outcome pathway	KeyEvent
Aop:622 - Calcineurin inhibitor induced nephrotoxicity leading to kidney failure	KeyEvent
Aop:624 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via insulin resistance-associated mitochondrial dysfunction	KeyEvent
Aop:627 - Increased 11β-Hydroxysteroid dehydrogenase type 1 activity leading to MASLD progression via lipogenesis-associated mitochondrial dysfunction	KeyEvent
Aop:636 - Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	KeyEvent
Aop:472 - DNA adduct formation leading to kidney failure	KeyEvent
Aop:645 - Binding and activation of AhR lead to cardiovascular aging	KeyEvent

Stressors

Name

Uranium

Nanoparticles and Micrometer
Particles

Cadmium

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

eukaryotic cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term

Scientific Term

Evidence Links

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
Drosophila melanogaster	Drosophila melanogaster	High	NCBI
Caenorhabditis elegans	Caenorhabditis elegans	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Male	High
Female	High

Mitochondrial dysfunction is a universal event occurring in cells of any species (Farooqui and Farooqui, 2012). Many invertebrate species (drosophila, C. elegans) are considered as potential models to study mitochondrial function. New data on marine invertebrates, such as molluscs and crustaceans and non-Drosophila species, are emerging (Martinez-Cruz et al., 2012). Mitochondrial dysfunction can be measured in animal models used for toxicity testing (Winklhofer and Haass, 2010; Waerzeggers et al., 2010) as well as in humans (Winklhofer and Haass, 2010).

- Revision of AOP3 (Project: [NP/EFSA/PREV/2024/02](#)): Endogenous ROS formation by complex I: In mammals, complex I is a dominant site of mitochondrial ROS, especially via RET. In plants (Senkler et al. 2017; Maldonado), mitochondria contain alternative NAD(P)H dehydrogenases and an alternative oxidase (AOX) that bypass Complex I and III. These pathways reduce ROS formation by preventing over-reduction of the ETC. Complex I still produces ROS, but generally less damaging due to AOX. Yeast: S. cerevisiae lacks a canonical Complex I entirely, relying instead on alternative NADH dehydrogenases. Consequently, mitochondrial ROS production from a Complex I-like source is absent. Other fungi with true Complex I (e.g., Neurospora crassa) do generate ROS similar to animals. **- Not endorsed**

Key Event Description

Mitochondrial dysfunction is a consequence of inhibition of the respiratory chain leading to oxidative stress.

Mitochondria can be found in all cells and are considered the most important cellular consumers of oxygen. Furthermore, mitochondria possess numerous redox enzymes capable of transferring single electrons to oxygen, generating the superoxide (O2-). Some mitochondrial enzymes that are involved in reactive oxygen species (ROS) generation include the electron-transport chain (ETC) complexes I, II and III; pyruvate dehydrogenase (PDH) and glycerol-3-phosphate dehydrogenase (GPDH). The transfer of electrons to oxygen, generating superoxide, happens mainly when these redox carriers are charged enough with electrons and the potential energy for transfer is elevated, like in the case of high mitochondrial membrane potential. In contrast, ROS generation is decreased if there are not enough electrons and the potential energy for the transfer is not sufficient (reviewed in Lin and Beal, 2006).

Cells are also able to detoxify the generated ROS due to an extensive antioxidant defence system that includes superoxide dismutases, glutathione peroxidases, catalase, thioredoxins, and peroxiredoxins in various cell organelles (reviewed in Lin and Beal, 2006). It is worth mentioning that, as in the case of ROS generation, antioxidant defences are also closely related to the redox and energetic status of mitochondria. If mitochondria are structurally and functionally healthy, an antioxidant defence mechanism balances ROS generation, and there is not much available ROS production. However, in case of mitochondrial damage, the antioxidant defence capacity drops and ROS generation takes over. Once this happens, a vicious cycle starts and ROS can further damage mitochondria, leading to more free-radical generation and further loss of antioxidant capacity. During mitochondrial dysfunction the availability of ATP also decreases, which is considered necessary for repair mechanisms after ROS generation.

A number of proteins bound to the mitochondria or endoplasmic reticulum (ER), especially in the mitochondria-associated ER membrane (MAM), are playing an important role of communicators between these two organelles (reviewed Mei et al., 2013). ER stress induces mitochondrial dysfunction through regulation of Ca2+ signaling and ROS production (reviewed Mei et al., 2013). Prolonged ER stress leads to release of Ca2+ at the MAM and increased Ca2+ uptake into the mitochondrial matrix, which induces Ca2+-dependent mitochondrial outer membrane permeabilization and apoptosis. At the same, ROS are produced by proteins in the ER oxidoreductin 1 (ERO1) family. ER stress activates ERO1 and leads to excessive production of ROS, which, in turn, inactivates SERCA and activates inositol-1,4,5- trisphosphate receptors (IP3R) via oxidation, resulting in elevated levels of cytosolic Ca2+, increased mitochondrial uptake of Ca2+, and ultimately mitochondrial dysfunction. Just as ER stress can lead to mitochondrial dysfunction, mitochondrial dysfunction also induces ER Stress (reviewed Mei et al., 2013). For example, nitric oxide disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca2+ flux which induce ER stress. Increased Ca2+ flux triggers loss of mitochondrial membrane potential (MMP), opening of mitochondrial permeability

transition pore (mPTP), release of cytochrome c and apoptosis inducing factor (AIF), decreasing ATP synthesis and rendering the cells more vulnerable to both apoptosis and necrosis (Wang and Qin, 2010).

Metal-induced Mitochondrial Dysfunction

Mitochondria are an important site of Ca^{2+} regulation and storage, taking up Ca^{2+} ions electrophoretically from the cytosol through a Ca^{2+} uniporter, which can then accumulate in the mitochondria (Roos et al., 2012; Orrenius et al., 2015). Similarities between calcium and metals, such as cadmium and lead, makes the entrance and accumulation of these metals into the mitochondria via calcium metals possible by mode of molecular mimicry (Mathews et al., 2013; Adiele et al., 2012). The outer mitochondrial membrane also contains the divalent metal transporter (DMT1), which allows for mitochondrial uptake of divalent metals such as Fe and Mn. When cells are under heavy metal-induced stress, DMT has been shown to be overexpressed in the mitochondrial membrane, making the mitochondria targets of metal toxicity and accumulation.

Heavy metal exposure in aerobic organisms increases ROS formation through redox cycling, where metals with different valence states (Fe, Cu, Cr, etc.) directly produce ROS as they are reduced by cellular antioxidants and then react with oxygen (Shaki et al., 2012; Shaki et al., 2013; Pourahmad et al., 2006; Santos et al., 2007). The production of highly reactive hydroxyl radicals under mitochondrial oxidative stress and in the presence of transition metals occurs via the Fenton reaction or Haber-Weiss reaction (Hancock et al., 2001; Valko et al., 2005; Adam-Vizi et al., 2010). Metals and ROS are capable of damaging mitochondrial DNA as well as mechanisms of DNA repair and proliferation arrest (Valko et al., 2005). Metals and ROS have the potential to directly damage mitochondrial membranes and structure by binding to and oxidizing membrane lipids and proteins. This structural damage can collapse the MMP and lead to the opening of the mPTP (Orrenius et al., 2015; Roos et al., 2012; Pourahmad et al., 2006). Uranium and mercury, for example, have both been shown to directly inhibit the mitochondrial electron transport chain and interfere with ATP production (Shaki et al., 2012; Roos et al., 2012). Furthermore, as previously mentioned, metals have been shown to inhibit ROS-detoxifying enzymes. By binding to these enzymes, metals can inhibit their antioxidant functions, and cause an accumulation of ROS and increased synthesis of more antioxidant enzymes in order to combat the oxidative stress (Blajszczak and Bonini, 2017).

Summing up: Mitochondria play a pivotal role in cell survival and cell death because they are regulators of both energy metabolism and apoptotic/necrotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002). The production of ATP via oxidative phosphorylation is a vital mitochondrial function (Kann and Kovács, 2007; Nunnari and Suomalainen, 2012). The ATP is continuously required for signalling processes (e.g. Ca^{2+} signalling), maintenance of ionic gradients across membranes, and biosynthetic processes (e.g. protein synthesis, heme synthesis or lipid and phospholipid metabolism) (Kang and Pervaiz, 2012), and (Green, 1998; McBride et al., 2006). Inhibition of mitochondrial respiration contributes to various cellular stress responses, such as deregulation of cellular Ca^{2+} homeostasis (Graier et al., 2007) and ROS production (Nunnari and Suomalainen, 2012; reviewed Mei et al., 2013). It is well established in the existing literature that mitochondrial dysfunction may result in: (a) an increased ROS production and a decreased ATP level, (b) the loss of mitochondrial protein import and protein biosynthesis, (c) the reduced activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, (d) the loss of the mitochondrial membrane potential, (e) the loss of mitochondrial motility, causing a failure to re-localize to the sites with increased energy demands (f) the destruction of the mitochondrial network, and (g) increased mitochondrial Ca^{2+} uptake, causing Ca^{2+} overload (reviewed in Lin and Beal, 2006; Graier et al., 2007), (h) the rupture of the mitochondrial inner and outer membranes, leading to (i) the release of mitochondrial pro-death factors, including cytochrome c (Cyt. c), apoptosis-inducing factor, or endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013), which eventually leads to apoptotic, necrotic or autophagic cell death (Wang and Qin, 2010). Due to their structural and functional complexity, mitochondria present multiple targets for various compounds.

How it is Measured or Detected

Mitochondrial dysfunction can be detected using isolated mitochondria, intact cells or cells in culture as well as in vivo studies. Such assessment can be performed with a large range of methods (revised by Brand and Nicholls, 2011) for which some important examples are given. All approaches to assess mitochondrial dysfunction fall into two main categories: the first assesses the consequences of a loss-of-function, i.e. impaired functioning of the respiratory chain and processes linked to it. Some assay to assess this have been described for KE1, with the limitation that they are not specific for complex I. In the context of overall mitochondrial dysfunction, the same assays provide useful information, when performed under slightly different assay conditions (e.g. without addition of complex III and IV inhibitors). The second approach assesses a 'non-desirable gain-of-function', i.e. processes that are usually only present to a very small degree in healthy cells, and that are triggered in a cell, in which mitochondria fail.

I. Mitochondrial dysfunction assays assessing a loss-of function.

1. Cellular oxygen consumption.

See KE1 for details of oxygen consumption assays. The oxygen consumption parameter can be combined with other endpoints to derive more specific information on the efficacy of mitochondrial function. One approach measures the ADP-to- O_2 ratio (the number of ADP molecules phosphorylated per oxygen atom reduced (Hinkle, 1995 and Hafner et al., 1990). The related P/O ratio is calculated from the amount of ADP added, divided by the amount of O_2 consumed while phosphorylating the added ADP (Ciapaite et al., 2005; Diepart et al., 2010; Hynes et al., 2006; James et al., 1995; von Heimbürg et al., 2005).

2. Mitochondrial membrane potential ($\Delta\psi_m$).

- **Revision of AOP3 (Project: [NP/EFSA/PREV/2024/02](https://npefsa.europa.eu/EFSA/PREV/2024/02)):** The mitochondrial membrane potential ($\Delta\psi_m$) is the electric

potential difference across the inner mitochondrial membrane. It requires a functioning respiratory chain in the absence of mechanisms that dissipate the proton gradient without coupling it to ATP production. Quantitative assessment of $\Delta\Psi_m$ in living cells is most commonly achieved through the use of cationic, lipophilic fluorescent probes that accumulate within the mitochondrial matrix in proportion to the electrochemical gradient (Leonard et al., 2014). Among these, tetramethylrhodamine derivatives such as TMRE (tetramethylrhodamine ethyl ester) and TMRM (tetramethylrhodamine methyl ester) are widely employed due to their reversible, potential-dependent distribution across the inner mitochondrial membrane (Scaduto and Grotyohann, 1999; Creed and McKenzie, 2019). When applied at non-quenching, nanomolar concentrations, these dyes allow linear and quantitative detection of $\Delta\Psi_m$, as fluorescence intensity directly correlates with mitochondrial polarization. Detection can be performed by flow cytometry for population-level quantification, by high-content microscopy for spatially resolved analysis, or by fluorescence plate readers for higher throughput (Wong and Cortopassi, 2002; Valdebenito and Dunchen, 2022). Quantitative interpretation requires the use of appropriate controls, typically involving treatment with protonophores such as FCCP or CCCP, which fully dissipate $\Delta\Psi_m$ and thereby establish baseline fluorescence, and inhibitors such as oligomycin or antimycin A to reveal different components of mitochondrial respiration. In parallel, dyes such as JC-1 are also used, though their ratiometric readout is less sensitive at low potentials and more prone to artifacts compared with TMRE or TMRM (Leonard et al., 2022). For accurate normalization, measurements are often corrected for cell number, mitochondrial content, or total protein, and fluorescence changes are expressed relative to maximal depolarization. In addition to chemical probes, genetically encoded sensors, such as mitochondria-targeted fluorescent proteins fused to potential-sensitive domains, provide complementary tools for $\Delta\Psi_m$ monitoring in live-cell and in vivo contexts (Leonard et al., 2022). - **Not endorsed**

3. Enzymatic activity of the electron transport system (ETS).

Determination of ETS activity can be done following Owens and King's assay (1975). The technique is based on a cell-free homogenate that is incubated with NADH to saturate the mitochondrial ETS and an artificial electron acceptor [I - (4 -iodophenyl) -3 - (4 -nitrophenyl) -5-phenylte trazolium chloride (INT)] to register the electron transmission rate. The oxygen consumption rate is calculated from the molar production rate of INT-formazan which is determined spectrophotometrically (Cammen et al., 1990).

4. ATP content.

For the evaluation of ATP levels, various commercially-available ATP assay kits are offered based on luciferin and luciferase activity. For isolated mitochondria various methods are available to continuously measure ATP with electrodes (Laudet 2005), with luminometric methods, or for obtaining more information on different nucleotide phosphate pools (e.g. Ciapaite et al., (2005).

- **Revision of AOP3 (Project: [NP/EFSA/PREV/2024/02](https://npefsa.europa.eu/2024/02)):**

Determination of mitochondrial ATP production based on extracellular flux analysis

The method is based on the detection of OCR (Oxygen Consumption Rate) that represents mitochondrial respiration as well as on the detection of ECAR (extracellular acidification rate) / proton efflux rate (PER): reflects extracellular acidification, a proxy for glycolysis (lactate release) plus contributions from $\text{CO}_2/\text{HCO}_3^-$. PER is preferred over raw ECAR since it corrects for CO_2 -derived acidification (Desousa et al., 2023; Espinosa et al., 2022). Application of inhibitors of individual complexes of the respiratory chain allows the detection of ATP-linked OCR: portion of oxygen consumption directly driving ATP synthesis (lost after ATP synthase inhibition) (Yoo et al., 2024). The proton leak & non-mitochondrial OCR represents remaining oxygen consumption after ATP synthase and electron transport chain inhibitor addition. The difference yields the ATP-coupled respiration component.

Calculation of mitochondrial ATP production

Mito ATP production rate (pmol ATP/min) = OCR_{ATP} (pmol O_2 /min) $\times 2 \times \text{P/O}$

OCR_{ATP} : ATP-coupled portion of OCR.

Factor 2: each O_2 molecule contains two oxygen atoms.

P/O ratio: number of ATP molecules synthesized per oxygen atom reduced. A mean P/O ≈ 2.75 is typically assumed (validated across many cell types but substrate- and condition-dependent) (Plitzko and Loesgen, 2018; Mookerjee et al., 2017; Motawe et al., 2024).

Limitations

P/O ratio varies by substrate (glucose vs. fatty acids), cell type, and conditions. Fixed values are approximations.

Non-mitochondrial oxygen consumption (oxidases, peroxidases, etc.) can confound OCR, hence use of ETC inhibitors.

PER vs. ECAR: CO_2 -driven acidification must be corrected to avoid overestimating glycolytic ATP.

Normalization: results are usually expressed per cell, protein content, DNA, or mitochondrial mass — interpretation depends on normalization method.

- **Not endorsed**

II. Mitochondrial dysfunction assays assessing a gain-of function.

1. Mitochondrial permeability transition pore opening (PTP).

The opening of the PTP is associated with a permeabilization of mitochondrial membranes, so that different compounds and cellular constituents can change intracellular localization. This can be measured by assessment of the translocation of cytochrome c, adenylate kinase or AIF from mitochondria to the cytosol or nucleus. The translocation can be assessed biochemically in cell fractions, by imaging approaches in fixed cells or tissues or by life-cell imaging of GFP fusion proteins (Single 1998; Modjtahedi 2006). An alternative approach is to measure the accessibility of cobalt to the mitochondrial matrix in a calcein fluorescence quenching assay in live permeabilized cells (Petronilli et al., 1999).

2. mtDNA damage as a biomarker of mitochondrial dysfunction.

Various quantitative polymerase chain reaction (QPCR)-based assays have been developed to detect changes of DNA structure and sequence in the mitochondrial genome. mtDNA damage can be detected in blood after low-level rotenone exposure, and the damage persists even after CI activity has returned to normal. With a more sustained rotenone exposure, mtDNA damage is also detected in skeletal muscle. These data support the idea that mtDNA damage in peripheral tissues in the rotenone model may provide a biomarker of past or ongoing mitochondrial toxin exposure (Sanders et al., 2014a and 2014b).

3. Generation of ROS and resultant oxidative stress.

a. General approach. Electrons from the mitochondrial ETS may be transferred 'erroneously' to molecular oxygen to form superoxide anions. This type of side reaction can be strongly enhanced upon mitochondrial damage. As superoxide may form hydrogen peroxide, hydroxyl radicals or other reactive oxygen species, a large number of direct ROS assays and assays assessing the effects of ROS (indirect ROS assays) are available (Adam-Vizi, 2005; Fan and Li 2014). Direct assays are based on the chemical modification of fluorescent or luminescent reporters by ROS species. Indirect assays assess cellular metabolites, the concentration of which is changed in the presence of ROS (e.g. glutathione, malonaldehyde, isoprostanes, etc.) At the animal level the effects of oxidative stress are measured from biomarkers in the blood or urine.

b. Measurement of the cellular glutathione (GSH) status. GSH is regenerated from its oxidized form (GSSG) by the action of an NADPH dependent reductase ($\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+$). The ratio of GSH/GSSG is therefore a good indicator for the cellular $\text{NADH}^+/\text{NADPH}$ ratio (i.e. the redox potential). GSH and GSSG levels can be determined by HPLC, capillary electrophoresis, or biochemically with DTNB (Ellman's reagent). As excess GSSG is rapidly exported from most cells to maintain a constant GSH/GSSG ratio, a reduction of total glutathione (GSH/GSSG) is often a good surrogate measure for oxidative stress.

c. Quantification of lipid peroxidation. Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA)-reactive compounds such as malondialdehyde generated from the decomposition of cellular membrane lipid under oxidative stress (Pryor et al., 1976). This method is quite sensitive, but not highly specific. A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies. The formation of F₂-like prostanoid derivatives of arachidonic acid, termed F₂-isoprostanes (IsoP) has been shown to be more specific for lipid peroxidation. A number of commercial ELISA kits have been developed for IsoPs, but interfering agents in samples requires partial purification before analysis. Alternatively, GC/MS may be used, as robust (specific) and sensitive method.

d. Detection of superoxide production. Generation of superoxide by inhibition of complex I and the methods for its detection are described by Grivennikova and Vinogradov (2014). A range of different methods is also described by BioTek (<http://www.biotek.com/resources/articles/reactive-oxygen-species.html>). The reduction of ferricytochrome c may be used to assess the rate of superoxide formation (McCord, 1968). Like in other superoxide assays, specificity can only be obtained by measurements in the absence and presence of superoxide dismutase. Chemiluminescent reactions have been used for their increased sensitivity. The most widely used chemiluminescent substrate is lucigenin. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical, and they become membrane impermeable after oxidation (trapping at site of formation). The best characterized of these probes are Hydro-Cy3 and Hydro-Cy5. generation of superoxide in mitochondria can be visualized using fluorescence microscopy with MitoSOX™ Red reagent (Life Technologies). MitoSOX™ Red reagent is a cationic derivative of dihydroethidium that permeates live cells and accumulates in mitochondria.

e. Detection of hydrogen peroxide (H₂O₂) production. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products in the presence of hydrogen peroxide (Zhou et al., 1997; Ruch et al., 1983). The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H₂O₂ form increasing amounts of fluorescent product (Tarpley et al., 2004).

Summing up, mitochondrial dysfunction can be measured by: • ROS production: superoxide (O₂⁻), and hydroxyl radicals (OH⁻) • Nitrosative radical formation such as ONOO⁻ or directly by: • Loss of mitochondrial membrane potential (MMP) • Opening of mitochondrial permeability transition pores (mPTP) • ATP synthesis • Increase in mitochondrial Ca²⁺ • Cytochrome c release • AIF (apoptosis inducing factor) release from mitochondria • Mitochondrial Complexes enzyme activity • Measurements of mitochondrial oxygen consumption • Ultrastructure of mitochondria using electron microscope and mitochondrial fragmentation measured by labelling with DsRed-Mito expression (Knott et al, 2008) Mitochondrial dysfunction-induced oxidative stress can be measured by: • Reactive carbonyls formations (proteins oxidation) • Increased 8-oxo-dG immunoreactivity (DNA oxidation) • Lipid peroxidation

(formation of malondialdehyde (MDA) and 4- hydroxynonenal (HNE) • 3-nitrotyrosine (3-NT) formation, marker of protein nitration • Translocation of Bid and Bax to mitochondria • Measurement of intracellular free calcium concentration ([Ca²⁺]_i): Cells are loaded with 4 μM fura-2/AM). • Ratio between reduced and oxidized form of glutathione (GSH depletion) (Promega assay, TB369; Radkowsky et al., 1986) • Neuronal nitric oxide synthase (nNOS) activation that is Ca²⁺-dependent. All above measurements can be performed as the assays for each readout are well established in the existing literature (e.g. Bal-Price and Brown, 2000; Bal-Price et al., 2002; Fujikawa, 2015; Walker et al., 1995). See also KE [Oxidative Stress, Increase](#)

Assay Type & Measured Content	Description	Dose Range Studied	Assay Characteristics (Length/Ease of use/Accuracy)
Rhodamine 123 Assay Measuring Mitochondrial membrane potential (MMP) and its collapse (Shaki et al., 2012)	Mitochondrial uptake of cationic fluorescent dye, rhodamine 123, is used for estimation of mitochondrial membrane potential. The fluorescence was monitored using Shimadzu RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively.	50, 100 and 500 μM of uranyl acetate	Short / easy Medium accuracy
TMRE Fluorescence Assay Measuring Mitochondrial permeability transition pore (mPTP) opening (Huser et al., 1998)	Laser scanning confocal microscopy in combination with the potentiometric fluorescence dye tetramethylrhodamine ethyl ester to monitor relative changes in membrane potential in single isolated cardiac mitochondria. The cationic dye distributes across the membrane in a voltage-dependent manner. Therefore, the large potential gradient across the inner mitochondrial membrane results in the accumulation of the fluorescent dye within the matrix compartment. Rapid depolarizations are caused by the opening of the transition pore.	1 μM cyclosporin A	Short / easy Low accuracy
GSH / GSSG Determination Assay Measuring cellular glutathione (GSH) status; ratio of GSH/GSSG (Owen & Butterfield, 2010; Shaki et al., 2013)	GSH and GSSG levels are determined biochemically with DTNB (Ellman's reagent). The developed yellow color was read at 412 nm on a spectrophotometer.	100 μM uranyl acetate	Short / easy Low accuracy
TBARS Assay Quantification of lipid peroxidation (Yuan et al., 2016)	MDA content, a product of lipid peroxidation, was measured using a thiobarbituric acid reactive substances (TBARS) assay. Briefly, the kidney cells were collected in 1 ml PBS buffer solution (pH 7.4) and sonicated. MDA reacts with thiobarbituric acid forming a colored product which can be measured at an absorbance of 532 nm.	200, 400, 800 μM uranyl acetate	Medium / medium High accuracy
Aequorin-based bioluminescence assay Increase in mitochondrial Ca ²⁺ influx (Pozzan & Rudolf, 2009)	Together with GFP, the aequorin moiety acts as Ca ²⁺ sensor <i>in vivo</i> , which delivers emission energy to the GFP acceptor molecule in a BRET (Bioluminescence Resonance Energy Transfer) process; the Ca ²⁺ can then be visualized with fluorescence microscopy.		Short / easy Low accuracy

Western blot & immunostaining analyses Measuring cytochrome c release (Chen et al., 2000)	Examining the redistribution of Cyto c in cytosolic and mitochondrial cellular fractions. Cells are homogenized and centrifuged, then prepared for immunoblots. Cellular fractions were washed in PBS and lysed in 1% NP-40 buffer. Cellular proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, probed using immunoblot analyses with antibodies specific to cyto c (6581A for Western and 65971A for immunostaining; Pharmingen)		Short / easy Medium accuracy
Quantikine Rat/Mouse Cytochrome c Immunoassay Measuring cytochrome c release (Shaki et al., 2012)	Cytochrome C release was measured a monoclonal antibody specific for rat/mouse cytochrome c was precoated onto the microplate. Seventy-five microliter of conjugate (containing monoclonal antibody specific for cytochrome c conjugated to horseradish peroxidase). After 2 h of incubation, the substrate solution (100 µl) was added to each well and incubated for 30 min. After 100 µl of the stop solution was added to each well; the optical density of each well was determined by the aforementioned microplate spectrophotometer set to 450 nm.		Short / easy Low accuracy
Membrane potential and cell viability - Flow Cytometry Measuring cytochrome c release (Kruidering et al., 1997)	“Dc and viability were determined by analyzing the R123 and propidium iodide fluorescence intensity with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser, with the Lysis software program (Becton Dickinson). R123 is a cationic dye that accumulates in the negatively charged inner side of the mitochondria. When the potential drops, less R123 accumulates in the mitochondria, which results in a lower fluorescence signal. The potential was measured as follows: at the indicated times, a 500-ml sample of the cell suspension was taken and transferred to an Eppendorf minivial. To this sample, 100 ml of 6 mM R123 in buffer D was added. After incubation for 10 min at 37°C, the cell suspension was centrifuged for 5 min at 80 3 g. The cell pellet was resuspended in 200 ml of buffer D, containing 0.2 mM R123 and 10 mM propidium iodide, to prevent loss of R123 and to stain nonviable cells, respectively. The samples were transferred to FACScan tubes and analyzed immediately. Analysis was performed at a flow rate of 60 ml/min. R123 fluorescence was detected by the FL1 detector with an emission detection limit below 560 nm. Propidium iodide fluorescence was detected by the FL3 detector, with emission detection above 620 nm. Per sample 3,000 to 5,000 cells were counted (Van de Water <i>et al.</i> , 1993)”		Short / easy Medium accuracy

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Event: 188: Neuroinflammation

Short Name: Neuroinflammation

Event Component

Process	Object	Action
brain inflammation	microglial cell	pathological

Process	Object	Action
brain inflammation	astrocyte	pathological

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	KeyEvent
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent
Aop:3 - Inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:374 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on brain cells (neuronal and non-neuronal) leads to neuroinflammation resulting in encephalitis	KeyEvent
Aop:429 - A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	KeyEvent
Aop:490 - Co-activation of IP3R and RyR leads to reduced IQ and increased socio-economic burden through non-cholinergic mechanisms	KeyEvent
Aop:471 - Neuron defect induced early behavioral change	KeyEvent
Aop:535 - Binding and activation of GPER leading to learning and memory impairments	KeyEvent
Aop:636 - Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	KeyEvent

Stressors

Name
SARS-CoV
Sars-CoV-2
Chemical
Virus
bacteria

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
zebrafish	Danio rerio	Low	NCBI
Macaca fascicularis	Macaca fascicularis	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
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Mixed High

Neuroinflammation is observed in human, monkey, rat, mouse, and zebrafish, in association with neurodegeneration or following toxicant exposure, or SARS-CoV-2 and other coronavirus infection. Some references (non-exhaustive list) are given below for illustration:

Human: Vennetti et al., 2006

Monkey (Macaca fascicularis): Charleston et al., 1994, 1996

Rat: Little et al., 2012; Zurich et al., 2002; Eskes et al., 2002

Mouse: Liu et al., 2012

Zebrafish: Xu et al., 2014.

Key Event Description

Neuroinflammation or brain inflammation differs from peripheral inflammation in that the vascular response and the role of peripheral bone marrow-derived cells are less conspicuous. The most easily detectable feature of neuroinflammation is activation of microglial cells and astrocytes. It is evidenced by changes in shape, increased expression of certain antigens, and accumulation and proliferation of the glial cells in affected regions (Aschner, 1998; Graeber & Streit, 1990; Monnet-Tschudi et al, 2007; Streit et al, 1999; Kraft and Harry, 2011; Claycomb et al., 2013). Upon stimulation by cytokines or inflammogens (e.g. from pathogens or from damaged neurons), both glial cell types activate inflammatory signalling pathways, which result in increased expression and/or release of inflammatory mediators such as cytokines, eicosanoids, and metalloproteinases (Dong & Benveniste, 2001), as well as in the production of reactive oxygen (ROS) and nitrogen species (RNS) (Brown & Bal-Price, 2003). Different types of activation states are possible for microglia and astrocytes, resulting in pro-inflammatory or anti-inflammatory signalling and other cellular functions (such as phagocytosis) (Streit et al., 1999; Nakajima and Kohsaka, 2004).

Therefore, neuroinflammation can have both neuroprotective/neuroreparative and neurodegenerative consequences (Carson et al., 2006 ; Monnet-Tschudi et al, 2007; Aguzzi et al., 2013 ; Glass et al., 2010). Under normal physiological conditions, microglial cells scan the nervous system for neuronal integrity (Nimmerjahn et al, 2005) and for invading pathogens (Aloisi, 2001; Kreutzberg, 1995; Kreutzberg, 1996; Rivest, 2009). They are the first type of cell activated (first line of defence), and can subsequently induce astrocyte activation (Falsig, 2008). Two distinct states of microglial activation have been described (Gordon, 2003; Kigerl et al, 2009; Maresz et al, 2008; Mosser & Edwards, 2008; Perego et al; Ponomarev et al, 2005; Moehle and West, 2015): The M1 state is classically triggered by interferon-gamma and/or other pro-inflammatory cytokines, and this state is characterized by increased expression of integrin alpha M (Itgam) and CD86, as well as the release of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6), and it is mostly associated with neurodegeneration. The M2 state is triggered by IL-4 and IL-13 (Maresz et al, 2008; Perego et al, 2011; Ponomarev et al, 2007) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes. The activation of astrocytes by microglia-derived cytokines or TLR agonists resembles the microglial M1 state (Falsig 2006). Although classification of the M1/M2 polarization of microglial cells may be considered as a simplification of authentic microglial reaction states (Ransohoff, 2016), a similar polarization of reactive astrocytes has been described recently Liddlelow et al., 2017): Interleukin-1 alpha (IL-1alpha), TNF and subcomponent q (C1q) released by activated microglial cells induce A1-reactive astrocytes, which lose the ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis and induce the death of neurons and oligodendrocytes.

Neuroinflammation and Brain development

During brain development, microglia are known to play a critical role as shapers of neural circuits, by providing trophic factors and by remodeling and pruning synapses (Rajendran and Paolicelli, 2018). In addition to playing a role in synaptic management, microglia are important for the pruning of dying neurons and in the clearance of debris ([Harry, 2013](#)). Microglia seem to affect also processes associated with neuronal proliferation and differentiation (Harry and

Kraft, 2012). Similarly to microglia, astrocytes have instructive roles in neurogenesis, gliogenesis, angiogenesis, axonal outgrowth, synaptogenesis, and synaptic pruning (Reemst et al., 2016).

The development-dependent reactivity of microglial cells and astrocytes is not well known. Ischemic insult appears to elicit similar microglial reactivity both during brain development and in adulthood ([Baburamani et al, 2014](#); [Leonardo & Pennypacker, 2009](#)). In contrast, treatment with lead acetate was previously shown to result in a more pronounced microglial and astrocyte reactivity in immature 3D rat brain cell cultures as compared to mature ones ([Zurich et al, 2002](#)). Astrocyte reactivity was also more pronounced in immature 3D rat brain cell cultures following paraquat exposure, whereas development-dependent differences in the phenotype of reactive microglia were observed (Sandström et al., 2017). This suggests that neuroinflammation is occurring during brain development and may express a different phenotype than in adulthood, and that dysfunction of microglia and astrocyte during brain development could contribute to neurodevelopmental disorders and potentially to late-onset neuropathology (Reemst et al., 2016).

Neuroinflammation in relation to COVID19

SARS-CoV-2 patients with moderate and severe COVID-19 presented an elevated plasma levels of glial fibrillary acidic protein (GFAP), which is known as biochemical indicator of glial activation (Kanberg et al., 2020).

How it is Measured or Detected

Neuroinflammation, i.e. the activation of glial cells can be measured by quantification of cellular markers (most commonly), or of released mediators (less common). As multiple activation states exist for the two main cell types involved, it is necessary to measure several markers of neuroinflammation:

- Microglial activation can be detected based on the increased numbers of labeled microglia per volume element of brain tissue (due to increase of binding sites, proliferation, and immigration of cells) or on morphological changes. A specific microglial marker, used across different species, is CD11b. Alternatively various specific carbohydrate structures can be stained by lectins (e.g. IB4). Beyond that, various well-established antibodies are available to detect microglia in mouse tissue (F4/80), phagocytic microglia in rat tissue (ED1) or more generally microglia across species (Iba1). Transgenic mice are available with fluorescent proteins under the control of the CD11b promoter to easily quantify microglia without the need for specific stains.
- The most frequently used astrocyte marker is GFAP (99% of all studies) (Eng et al., 2000). This protein is highly specific for astrocytes in the brain, and antibodies are available for immunocytochemical detection. In neuroinflammatory brain regions, the stain becomes more prominent, due to an upregulation of the protein, a shape change/proliferation of the cells, and/or better accessibility of the antibody. Various histological quantification approaches can be used. Occasionally, alternative astrocytic markers, such as vimentin or the S100beta protein, have been used for staining of astrocytes (Struzynska et al., 2007). Antibodies for complement component 3 (C3), the most characteristic and highly upregulated marker of A1 neurotoxic reactive astrocytes are commercially available.
- All immunocytochemical methods can also be applied to cell culture models.
- In patients, microglial accumulation can be monitored by PET imaging, using [11C]-PK 11195 as a microglial marker (Banati et al., 2002).
- Activation of glial cells can be assessed in tissue or cell culture models also by quantification of sets of activation markers. This can for instance be done by PCR quantification of inflammatory factors, by measurement of the respective mediators, e.g. by ELISA-related immuno-quantification. Such markers include:
 - Pro- and anti-inflammatory cytokine expression (IL-1 β ; TNF- α , IL-6, IL-4); or expression of immunostimulatory proteins (e.g. MHC-II)
 - Itgam, CD86 expression as markers of M1 microglial phenotype
 - Arg1, MRC1, as markers of M2 microglial phenotype

For descriptions of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014

Regulatory example using the KE

Measurement of glial fibrillary acidic protein (GFAP) in brain tissue, whose increase is a marker of astrocyte reactivity, is required by the US EPA in rodent toxicity studies for fuel additives (40 CFR 79.67). It has been used on rare occasions for other toxicant evaluations.

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Event: 386: Decrease of neuronal network function

Short Name: Neuronal network function, Decreased

Event Component

Process	Object	Action
synaptic signaling		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1	KeyEvent
Aop:90 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony loss/failure 2	KeyEvent
Aop:54 - Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:405 - Organo-Phosphate Chemicals induced inhibition of AChE leading to impaired cognitive function	KeyEvent

AOP ID and Name	Event Type
Aop:429 - A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	KeyEvent
Aop:501 - Excessive iron accumulation leading to neurological disorders	KeyEvent
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent
Aop:522 - Estrogen antagonism leading to increased risk of autism-like behavior	KeyEvent
Aop:533 - Retinoic acid receptor antagonism during neurodevelopment leading to impaired learning and memory	KeyEvent
Aop:610 - Decreased thyroid hormone levels in the brain regulated via transport, metabolism and TR activation leading to decreased cognition and motor function	KeyEvent
Aop:636 - Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI
cat	Felis catus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

In vitro studies in brain slices applying electrophysiological techniques showed significant variability among species (immature rats, rabbits and kittens) related to synaptic latency, duration, amplitude and efficacy in spike initiation (reviewed in Erecinska et al., 2004).

Key Event Description

Biological state: There are striking differences in neuronal network formation and function among the developing and mature brain. The developing brain shows a slow maturation and a transient passage from spontaneous, long-duration action potentials to synaptically-triggered, short-duration action potentials.

Furthermore, at this precise developmental stage the neuronal network is characterised by "hyperexcitability", which is related to the increased number of local circuit recurrent excitatory synapses and the lack of γ -amino-butyric acid A (GABAA)-mediated inhibitory function that appears much later. This "hyperexcitability" disappears with maturation when pairing of the pre- and postsynaptic partners occurs and synapses are formed generating population of postsynaptic potentials and population of spikes followed by developmental GABA switch. Glutamatergic neurotransmission is dominant at early stages of development and NMDA receptor-mediated synaptic currents are far

more times longer than those in maturation, allowing more calcium to enter the neurons. The processes that are involved in increased calcium influx and the subsequent intracellular events seem to play a critical role in establishment of wiring of neural circuits and strengthening of synaptic connections during development (reviewed in Erecinska et al., 2004). Neurons that do not receive glutamatergic stimulation are undergoing developmental apoptosis.

During the neonatal period, the brain is subject to profound alterations in neuronal circuitry due to high levels of synaptogenesis and gliogenesis. For example, in neuroendocrine regions such as the preoptic area-anterior hypothalamus (POA-AH), the site of gonadotropin-releasing hormone (GnRH) system is developmentally regulated by glutamatergic neurons. The changes in the expression of the N-methyl-D-aspartate (NMDA) receptor subunits NR1 and NR2B system begin early in postnatal development, before the onset of puberty, thereby playing a role in establishing the appropriate environment for the subsequent maturation of GnRH neurons (Adams et al., 1999).

Biological compartments: Neural network formation and function happen in all brain regions but it appears to onset at different time points of development (reviewed in Erecinska et al., 2004). Glutamatergic neurotransmission in hippocampus is poorly developed at birth. Initially, NMDA receptors play important role but the vast majority of these premature glutamatergic synapses are “silent” possibly due to delayed development of hippocampal AMPA receptors. In contrast, in the cerebral cortex the maturation of excitatory glutamatergic neurotransmission happens much earlier. The “silent” synapses disappear by PND 7-8 in both brain regions mentioned above.

There is strong evidence suggesting that NMDA receptor subunit composition controls synaptogenesis and synapse stabilization (Gambrell and Barria, 2011). It is established fact that during early postnatal development in the rat hippocampus, synaptogenesis occurs in parallel with a developmental switch in the subunit composition of NMDA receptors from NR2B to NR2A. It is suggested that early expression of NR2A in organotypic hippocampal slices reduces the number of synapses and the volume and dynamics of spines. In contrast, overexpression of NR2B does not affect the normal number and growth of synapses. However, it does increase spine motility, adding and retracting spines at a higher rate. The C terminus of NR2B, and specifically its ability to bind CaMKII, is sufficient to allow proper synapse formation and maturation. Conversely, the C terminus of NR2A was sufficient to stop the development of synapse number and spine growth. These results indicate that the ratio of synaptic NR2B over NR2A controls spine motility and synaptogenesis, and suggest a structural role for the intracellular C terminus of NR2 in recruiting the signalling and scaffolding molecules necessary for proper synaptogenesis. Interestingly, it was found that genetic deletion of NR3A accelerates glutamatergic synaptic transmission, as measured by AMPAR-mediated postsynaptic currents recorded in hippocampal CA1. Consistent, the deletion of NR3A accelerates the expression of the glutamate receptor subunits NR1, NR2A, and GluR1 suggesting that glutamatergic synapse maturation is critically dependent upon activation of NMDA-type glutamate receptors (Henson et al., 2012).

General role in biology: The development of neuronal networks can be distinguished into two phases: an early ‘establishment’ phase of neuronal connections, where activity-dependent and independent mechanisms could operate, and a later ‘maintenance’ phase, which appears to be controlled by neuronal activity (Yuste and Sur, 1999). These neuronal networks facilitate information flow that is necessary to produce complex behaviors, including learning and memory (Mayford et al., 2012).

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

In vivo: The recording of brain activity by using electroencephalography (EEG), electrocorticography (ECoG) and local field potentials (LFP) assists towards the collection of signals generated by multiple neuronal cell networks. Advances in computer technology have allowed quantification of the EEG and expansion of quantitative EEG (qEEG) analysis providing a sensitive tool for time-course studies of different compounds acting on neuronal networks' function (Binienda et al., 2011). The number of excitatory or inhibitory synapses can be functionally studied at an electrophysiological level by examining the contribution of glutamatergic and GABAergic synaptic inputs. The number of them can be determined by variably clamping the membrane potential and recording excitatory and inhibitory postsynaptic currents (EPSCs or IPSCs) (Liu, 2004).

In vitro: Microelectrode array (MEA) recordings are also used to measure electrical activity in cultured neurons (Keefer et al., 2001; Gramowski et al., 2000; Gopal, 2003; Johnstone et al., 2010). MEAs can be applied in high throughput platforms to facilitate screening of numerous chemical compounds (McConnell et al., 2012). Using selective agonists and antagonists of different classes of receptors their response can be evaluated in a quantitative manner (Novellino et al., 2011; Hogberg et al., 2011).

Patch clamping technique can also be used to measure neuronal network activity. In some cases, if required, planar patch clamping technique can also be used to measure neuronal networks activity (e.g., Bosca et al., 2014).

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List of Adverse Outcomes in this AOP

[Event: 2416: human amyotrophic lateral sclerosis \(ALS\)](#)

Short Name: ALS

Event Component

Process	Object	Action
Amyotrophic lateral sclerosis	adult central nervous system	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:636 - Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	AdverseOutcome

Biological Context

Level of Biological Organization

Level of Biological Organization

Individual

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability**Life Stage Evidence**

Adult High

Sex Applicability**Sex Evidence**

Male High

Female High

Taxonomic Applicability

- **Primary Taxon:** *Homo sapiens* (NCBI Taxon ID: 9606)
- **Secondary / Model Taxa:** *Mus musculus* (House mouse, NCBI Taxon ID: 10090), *Rattus norvegicus* (Norway rat, NCBI Taxon ID: 10116)
- **Scientific Rationale:** While human motor neuron physiology, neurofilament composition, and lifespan represent the primary domain, transgenic and knock-in rodent models harboring human ALS-associated mutations (e.g., *SOD1* G93A, mutant *TARDBP*, *FUS*, *C9orf72* repeat expansions) exhibit overlapping phenotypic features of motor neuron degeneration, axonal pathology, and muscle denervation.

Life Stage Applicability

- **Domain:** Adult, Aging / Senescent (Late adulthood)
- **Scientific Rationale:** ALS is primarily a late-onset neurodegenerative disorder. The median age of symptom onset in sporadic ALS is typically between 55 and 75 years, although familial forms with high-penetrance genetic mutations can present earlier in young to mid-adulthood (30s to 50s).

Sex Applicability

- **Domain:** Unspecific (Applies to both sexes)
- **Scientific Rationale:** ALS affects both males and females. A slight male predominance is clinically observed in sporadic ALS (male-to-female ratio of approximately 1.2:1 to 1.5:1), but the underlying cellular and molecular mechanisms of neurodegeneration operate across both sexes.

Key Event Description

Irreversible motor neuron loss results in widespread denervation of neuromuscular junctions (NMJs), leading to progressive neurogenic skeletal muscle atrophy, fasciculations, and spastic/flaccid paralysis. As degeneration spreads, loss of bulbar and respiratory musculature (notably the diaphragm and intercostal muscles) causes dysarthria, dysphagia, and progressive ventilatory failure. Without mechanical respiratory intervention, this condition typically leads to fatal hypoventilation within 2 to 5 years of clinical onset.

How it is Measured or Detected

- **Clinical Rating Scale (ALSFERS-R):**
 - Serial assessment using the Revised ALS Functional Rating Scale (0–48 score), evaluating bulbar, fine motor, gross motor, and respiratory domains.
- **Pulmonary Function Testing:**
 - Longitudinal monitoring of forced vital capacity (%FVC in seated and supine positions), slow vital capacity (SVC), and sniff nasal inspiratory pressure (SNIP) to quantify diaphragmatic weakness.
- **Electromyography (Needle EMG):**
 - Demonstration of acute active denervation (fibrillation potentials, positive sharp waves) and chronic reinnervation changes (neurogenic motor unit action potentials) according to the Gold Coast and Awaji diagnostic criteria.
- **Skeletal Muscle Biopsy Histology:**
 - Verification of neurogenic atrophy characterized by grouped atrophy, small angulated esterase-positive fibers, and target fibers.

Regulatory Significance of the AO

The development of ALS represents an apical neurotoxicological endpoint of paramount regulatory significance, falling directly under international safety assessment and human health protection frameworks:

1. Neurotoxicity Hazard Identification and Characterization:

- Severe, irreversible neurodegeneration and motor system failure correspond directly to regulatory definitions of cumulative neurotoxicity and delayed neurotoxicity under chemical and pharmaceutical testing frameworks (e.g., OECD Test Guidelines for Neurotoxicity, US EPA Health Effects Test Guidelines, and ICH S7A/S7B safety pharmacology guidelines).

2. Support for Integrated Approaches to Testing and Assessment (IATA):

- Defining the AO and upstream Key Events facilitates the transition toward New Approach Methodologies (NAMs)—such as human iPSC-derived motor neuron-glia coculture assays, microfluidic neuromuscular junction models, and microelectrode array (MEA) electrophysiology. This enables mechanistic chemical screening and hazard ranking without sole reliance on long-term animal studies.

3. Identification of Chemical and Environmental Risk Factors:

- Regulatory agencies evaluate environmental contaminants, occupational neurotoxins (e.g., heavy metals, organophosphates, solvents), and xenobiotics for their potential to trigger or accelerate motor neuron death via proteostasis failure, oxidative stress, and mitochondrial impairment, informing occupational exposure limits and environmental safety thresholds.

4. Pharmaceutical and Biotherapeutic Safety:

- In drug discovery and preclinical safety evaluations, monitoring intermediate biomarkers aligned with this AO (e.g., elevated biofluid neurofilament light chain [NfL], cryptic exon inclusion) enables early de-risking of therapeutics that could inadvertently induce off-target axonal degeneration or disrupt RNA-binding protein homeostasis.

References

- Cedarbaum, J. M., et al. (1999). The ALSFRS-R: a revised ALS functional rating scale that incorporates assessments of respiratory function. *Journal of the Neurological Sciences*, 169(1-2), 13-21.
- Shefner, J. M., et al. (2020). A proposal for new diagnostic criteria for ALS: The Gold Coast Criteria. *Clinical Neurophysiology*, 131(8), 1975-1978.
- de Carvalho, M., et al. (2008). Electrodiagnostic criteria for diagnosis of ALS. *Clinical Neurophysiology*, 119(3), 497-503.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

[Relationship: 3496: Increase, ROS leads to Increase, Mitochondrial dysfunction](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Mitochondrial complex inhibition leading to liver injury	adjacent	High	High
Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	adjacent	High	

[Relationship: 3767: Increase, Mitochondrial dysfunction leads to Neuroinflammation](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	adjacent	High	

Relationship: 2500: Neuroinflammation leads to Neuronal network function, Decreased**AOPs Referencing Relationship**

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	adjacent	High	
Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	adjacent	High	

Relationship: 3768: Neuronal network function, Decreased leads to ALS**AOPs Referencing Relationship**

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increase in reactive oxygen species (ROS) leading to human amyotrophic lateral sclerosis (ALS)	adjacent	High	