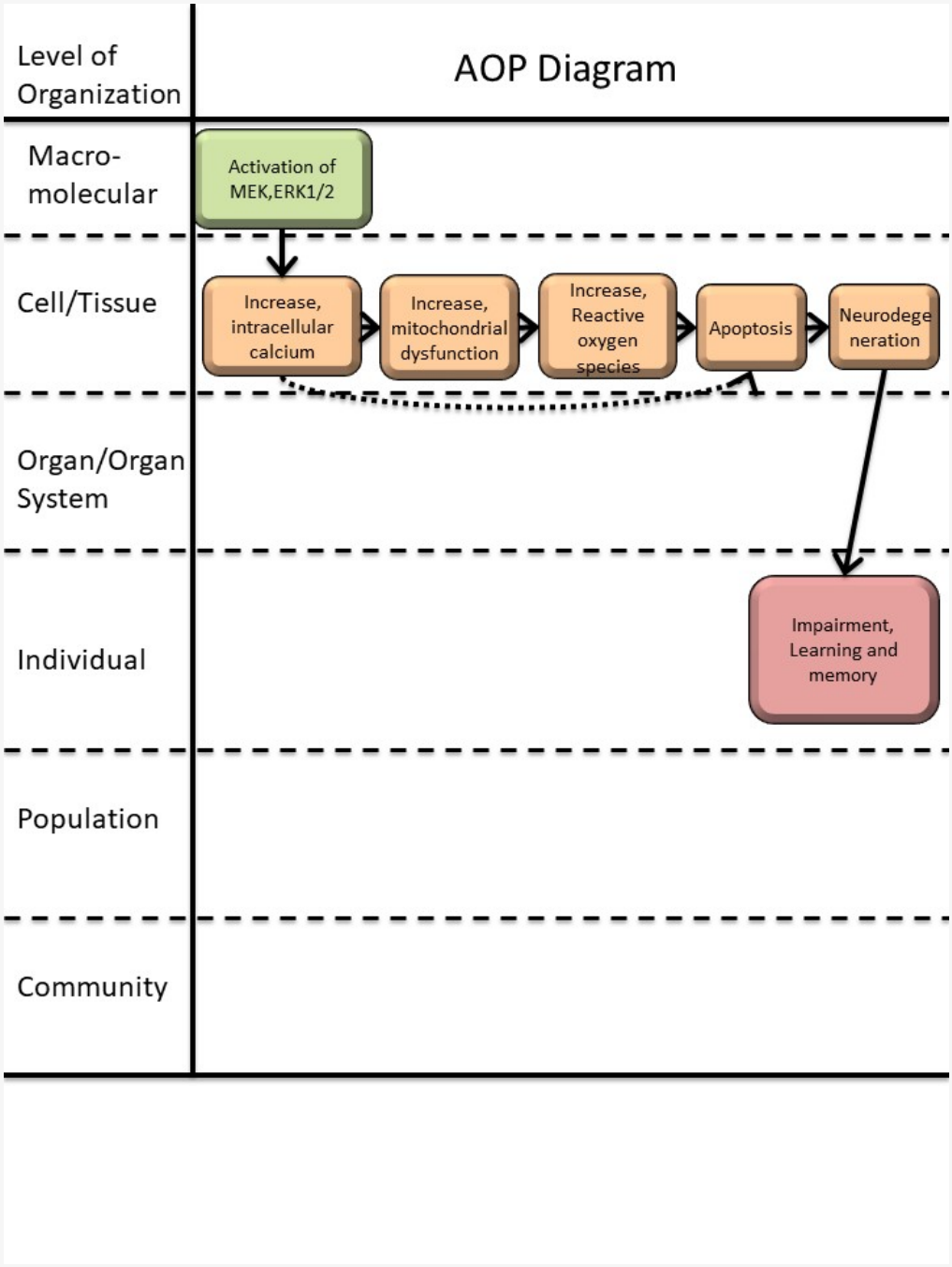
AOP ID and Title:

AOP 500: Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis
Short Title: MEK-ERK1/2 activation leading to deficits in learning and cognition via ROS

Graphical Representation



Authors

Of the originating work:

Katherine von Stackelberg *(Harvard Center for Risk Analysis, Boston, MA, USA.) (Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA.)*

Elizabeth Guzy *(Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA.)*

Tian Chu *(Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA.)*

Birgit Claus Henn *(Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA.) (Department of Environmental Health, Boston University School of Public Health, Boston, MA, USA.)*

Of the content populated in the AOP-Wiki:

Travis Karschnik *(General Dynamics Information Technology, Duluth, MN, USA.)*

Status

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Abstract

Metal mixture activation of ERK1/2 and JNK1/2 in astrocytes leads to increased Ca2+ release (Asit Rai et al., 2010). Alterations to calcium, an essential nutrient which is required in multiple cellular and physiological functions, such as cell adhesion, signal transduction, and neurotransmission can be expected to have downstream effects in those functions (Antonio et al., 2002). While a variety of stimuli can trigger opening of the mitochondrial transition pore and cause apoptosis, a sustained intracellular increase in Ca2+ is one of the better-known triggers (Mattson 2000). Mitochondria play a role in stress responses and can produce ROS when damaged. Mitochondria are indeed a major source of ROS (Yan et al., 2013). Unchecked, excessive ROS can lead to the destruction of cellular components including lipids, protein, and DNA, and ultimately cell death via apoptosis or necrosis (Kannan and Jain 2000). Aberrant apoptosis has been implicated in the pathogenesis of neurodegeneration (Okouchi et al., 2007). It is well accepted that impairment of cell function or cell loss (neurodegeneration) in hippocampus will interfere with memory processes, since the hippocampus plays a key role in memory (Barker and Warburton 2011).

MEK-ERK1/2 is important in understanding uptake of metals into the brain and its relationship to deficits in learning and cognition from exposure to metals commonly detected at Superfund sites including lead, cadmium, manganese, and arsenic. Current risk assessment guidance dictates a largely chemical-by-chemical evaluation of exposures and risks, which fails to adequately address potential interactions with other chemicals, nonchemical stressors, and genetic factors. Cumulative risk assessment methods and approaches are evolving to meet regulatory needs (MacDonell et al., 2013; Backhaus and Faust 2012; IPCS 2009), but significant challenges remain. As our understanding of complex exposures and interactions continues to grow, synthesis and integration across disciplines and studies focused on different aspects of the environmental fate–exposure–toxicology–health outcome continuum are required to assess the likelihood of adverse effects and to support cumulative risk assessment. Environmental exposures are virtually always to complex mixtures (von Stackelberg et al., 2015).

Background

An examination of neurodevelopmental disorders and subclinical effects using multi-domain global neurodevelopment assessments is warranted as they can have profound population level implications. In the context of neurotoxicity, neurodevelopmental pathways in the developing human brain are not fully understood (Schubert et al., 2013; Bal-Price et al., 2015) although there are a number of commonly observed phenomena which may take part in those pathways e.g. changes in intracellular calcium, ROS generation, apoptosis, and neurotransmitter disruption. This AOP highlights a specific set of response-response relationships using a subset of those commonly observed phenonema related to metals and metal mixture exposures leading to deficits in learning and cognition.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	2146	Activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase 1/2	Activation of MEK, ERK1/2
	KE	1339	Increase, intracellular calcium	Increase, intracellular calcium
	KE	177	N/A, Mitochondrial dysfunction 1	N/A, Mitochondrial dysfunction 1
	KE	1115	Increased, Reactive oxygen species	Increased, Reactive oxygen species
	KE	1262	Apoptosis	Apoptosis
	KE	352	N/A, Neurodegeneration	N/A, Neurodegeneration

Sequence	Type	Event ID	Title	Short name
	AO	34	Impairment, Learning and memory	Impairment, Learning and memory

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase 1/2	adjacent	Increase, intracellular calcium	Not Specified	Not Specified
Increase, intracellular calcium	adjacent	N/A, Mitochondrial dysfunction 1	Not Specified	Not Specified
N/A, Mitochondrial dysfunction 1	adjacent	Increased, Reactive oxygen species	Not Specified	Not Specified
Increased, Reactive oxygen species	adjacent	Apoptosis	Not Specified	Not Specified
Apoptosis	adjacent	N/A, Neurodegeneration	Not Specified	Not Specified
N/A, Neurodegeneration	adjacent	Impairment, Learning and memory	Not Specified	Not Specified

Stressors

Name	Evidence
Heavy metals (cadmium, lead, copper, iron, nickel)	
Lead	
Arsenic	
Cadmium	
Manganese	

Overall Assessment of the AOP

1. Support for Biological Plausibility of KERs	Defining Question	High (Strong)	Moderate	Low (Weak)
	Is there a mechanistic relationship between KE_{up} and KE_{down} consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs , but the structural or functional relationship between them is not understood.
Relationship 2942: Activation of MEK, ERK1/2 (2146) leads to Increase, intracellular calcium (1339)	**Moderate** Empirical evidence indicates a complex relationship between MEK, ERK1/2 activation and inhibition and Ca2+ response including Ca2+ feeding back into a ERK1/2 activation. This relationship appears to vary across species and cell type.			
Relationship 3140: Increase, intracellular calcium (1339) leads to N/A, Mitochondrial dysfunction 1 (177)	**Moderate** There are both accepted associations between these two KEs and empirical evidence but the current state of understanding falls short of extensive.			
Relationship 3141: N/A, Mitchondrial dysfunction 1 (177) leads to Increased, Reactive oxygen	**High** This relationship has been studied in humans and human-model rodents extensively related to age-related diseases.			

species (1115)	
Relationship 2966: Increased, Reactive oxygen species (1115) leads to Apoptosis (1262)	**High** This is a well-studied relationship across taxa where modulation of ROS and its effect on subsequent apoptosis has been examined.
Relationship 2967: Apoptosis (1262) leads to N/A, Neurodegeneration (352)	**High** This relationship has been studied in humans and human-model rodents extensively related to age-related diseases.
Relationship 1069: N/A, Neurodegeneration (352) leads to Impairment, Learning and memory (341)	**High** This relationship has been studied in humans and human-model rodents extensively related to age-related diseases.
Relationship 2968: Increase, intracellular calcium (1339) leads to Apoptosis (1262)	

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Mus musculus	Mus musculus	Moderate	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Life Stage

Life stages applicable to this AOP encompass the full life cycle. Many of the key events are measured in pregnant females with the adverse outcome (impairment, learning and memory) measured at all life stages.

Taxonomic Applicability

Most evidence for this AOP is derived from rodents and humans where rodents were selected with their ability to model human responses.

Sex Applicability

This AOP is applicable to all sexes.

Essentiality of the Key Events

2. Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE 2146: Activation	**Moderate**			

of MEK, ERK1/2	MEK, ERK1/2 activation is fundamental in delivering signals which regulate the cell cycle, proliferation, differentiation, adhesion, and more. Disruptions in this activation have wide reaching effects however, there is evidence that downstream KEs can also activate this KE.
KE 1339: Increase, intracellular calcium	**High** Calcium, as a primary intracellular messenger in neurons and regulator of cell responses to stress has been shown to play an integral role in subsequent KEs.
KE 177: N/A, Mitchondrial dysfunction 1	**High** The ubiquity and role of mitochondria in cell function is such that changes in this KE necessitate changes in downstream KEs.
KE 1115: Increased, Reactive oxygen species	**High** ROS has been shown to mediate apoptosis across taxa with changes in ROS levels affecting subsequent apoptosis.
KE 1262: Apoptosis	**High** Unregulated apoptosis has been shown to affect neurodegeneration and eventual learning and memory tasks in human and rodent models.
KE 352: N/A, Neurodegeneration	**High** Neurodegeneration has been causally linked to learning and memory tasks in human and rodent models.
KE 341: Impairment, Learning and memory	**N/A**
AOP 500	**High/Moderate** There is evidence for manipulation of downstream KEs based on manipulation of upstream KEs in multiple KERs.

Weight of Evidence Summary

3. Empirical Support for KERs	Defining Questions	High (Strong)	Moderate	Low (Weak)
	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup > than that for KEdown? Inconsistencies?	if there is dependent change in both events following exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance) and no or few data gaps or conflicting data	if there is demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with the expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	if there are limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all), and/or lacking evidence of temporal or dose-response concordance, or identification of significant inconsistencies in empirical support across taxa and species that don't align with the expected pattern for the hypothesised AOP
Relationship 2942: Activation of MEK, ERK1/2 (2146) leads to Increase, intracellular calcium (1339)	**Moderate** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure. Heavy metals like cadmium can complicate issues related to calcium levels since the metal itself can act in place of calcium in cell function.			
Relationship 3140: Increase, intracellular calcium (1339) leads to N/A, Mitchondrial dysfunction 1 (177)	**Moderate** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures. Some inconsistency was documented in the relationship between the two events regarding which preceded the other in different taxa and cell types. Heavy metals like cadmium can complicate issues related to calcium levels since the metal itself can act in place of calcium in cell function.			
Relationship 3141: N/A, Mitchondrial dysfunction 1 (177) leads to Increased,	**High** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events			

Reactive oxygen species (1115)	following exposure.
Relationship 2966: Increased, Reactive oxygen species (1115) leads to Apoptosis (1262)	**High** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure.
Relationship 2967: Apoptosis (1262) leads to N/A, Neurodegeneration (352)	**High** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure. There are also numerous studies investigating this relationship in the context of neurodegenerative diseases.
Relationship 1069: N/A, Neurodegeneration (352) leads to Impairment, Learning and memory (341)	**High** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure. There are also numerous studies investigating this relationship in the context of neurodegenerative diseases.
Relationship 2968: Increase, intracellular calcium (1339) leads to Apoptosis (1262)	**High** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure.

Considerations for Potential Applications of the AOP (optional)

Developmental neurotoxicity (DNT) is an adverse outcome of concern to multiple regulatory agencies. In vitro screening assays for MEK-ERK1/2 activation would not be recommended as a direct alternative or replacement to established DNT assays like OECD Test No. 426 (OECD 2007). However, detection of MEK-ERK1/2 activation in neuronal cell types may be used to prioritize chemicals with potential to elicit neurotoxicity and flag them for testing in ortogonal assays for evaluating DNT, including proposed alternative test methods (Bal-Price et al. 2018; Crofton et al 2022).

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Appendix 1

List of MIEs in this AOP

Event: 2146: Activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase 1/2

Short Name: Activation of MEK, ERK1/2

Key Event Component

Process	Object	Action
kinase activity	astrocyte	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	MolecularInitiatingEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

astrocyte

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	Moderate	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Mixed	Moderate

Key Event Description

ERK1 and ERK2 are proteins of 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates (Boulton et al., 1990; 1991). The two phosphoacceptor sites, tyrosine and threonine, which are phosphorylated to activate the kinases, are separated by a glutamate residue in both ERK1 and ERK2 to give the motif TEY in the activation loop (Payne et al., 1991). Both are ubiquitously expressed, although their relative abundance in tissues is variable. For example, in many immune cells ERK2 is the predominant species, while in several cells of neuroendocrine origin they may be equally expressed (Gray Pearson and others 2001). They are stimulated to some extent by a vast number of ligands and cellular perturbations, with some cell type specificity (Lewis et al., 1998). In fibroblasts (the cell type in which the generalizations about their behavior and functions have been developed) they are activated by serum, growth factors, cytokines, certain stresses, ligands for G protein-coupled receptors (GPCRs), and transforming agents, to name a few (Gray Pearson and others 2001). They are highly expressed in postmitotic neurons and other highly differentiated cells (Boulton et al., 1991). In these cells they are often involved in adaptive responses such as long-term potentiation (English and Sweatt 1996; Atkins et al., 1998; Rossi-Arnaud et al., 1997).

How it is Measured or Detected

Western blotting and immunoblotting.

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List of Key Events in the AOP

Event: 1339: Increase, intracellular calcium

Short Name: Increase, intracellular calcium

Key Event Component

Process	Object	Action
calcium amount	calcium(2+)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:214 - Network of SSRIs (selective serotonin reuptake inhibitors)	KeyEvent
Aop:226 - SSRI (Selective serotonin reuptake inhibitor) to hypertension	KeyEvent
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate
Birth to < 1 month	Moderate

Sex Applicability

Sex	Evidence
Mixed	Moderate

Key Event Description

Calcium is arguably the most versatile and important intracellular messenger in neurons (Berridge et al., 2000). Interestingly, although calcium may often promote neuronal death, it can also activate pathways that promote survival. For example, calcium can promote survival through a pathway involving activation of protein kinase B (PKB/Akt) by calcium/calmodulin-dependent protein kinase (Yano et al., 1998). Calcium is a prominent regulator of cellular responses to stress, activating transcription through the cyclic-AMP response element-binding protein (CREB), which can promote neuron survival in experimental models of developmental cell death (Hu et al., 1999). Calcium can also activate a rapid neuroprotective signalling pathway in which the calcium-activated actin-severing protein gelsolin induces actin depolymerization, resulting in suppression of calcium influx through membrane NMDA (N-methyl-d-aspartate) receptors and voltage-dependent calcium channels (Furukawa et al., 1997). This may occur through

intermediary actin-binding proteins that interact with NMDA receptor and calcium channel proteins. Finally, signals such as calcium and secreted amyloid precursor protein-α (sAPP-α), which increase cyclic GMP production, can induce activation of potassium channels and the transcription factor NF-κB, and thereby increase resistance of neurons to excitotoxic apoptosis (Furukawa et al., 1996).

How it is Measured or Detected

An increase in $[Ca^{2+}]_i$ was measured using Fluo3 AM as an indicator dye after the addition of metals (single or in mixture) to the culture wells following an optimized protocol (Arey et al., 2022). The fluorescent signals were read by fluorescence imaging plate reader Synergy HT (BioTek, Winooski, VT) (Rai and others 2010).

Briefly, Ca2+ levels in human astrocytes were monitored by fluorescence microscopy using the Ca2+ indicator fluo-4. Slices were incubated with fluo-4-AM (2–5 μL of 2 mM dye were dropped over the tissue, attaining a final concentration of 2–10 μM and 0.01% of pluronic) and Sulforhodamine 101 (100 μM) for 30–60 min at room temperature (Navarrete and others 2013). In these conditions, most of the Fluo-4-loaded cells were astrocytes as indicated by their SR101 staining (Nimmerjahn et al., 2004; Dombeck et al., 2007; Kafitz et al., 2008; Takata and Hirase 2008), and confirmed in some cases by their electrophysiological properties. Astrocytes were imaged with an Olympus FV300 laser-scanning confocal microscope or a CCD camera (Retiga EX) attached to the Olympus BX50WI microscope (Navarrete and others 2013).

Diversity of endogenous Ca2+ activity in a mature hippocampal astrocyte in situ: Ca2+ signals in cell body and processes are different. (A) Cumulative Ca2+ activity recorded in an astrocyte over a 165 s period revealed by the calcium indicator Fluo4-AM. The visible boundaries of the astrocyte are shown in white. Note the different intensities of spatially-confined local activity in the astrocyte cell body (s), primary process (p1) stemming from the soma and secondary processes (p2) branching from a primary process. Intensity of the normalized cumulative activity is expressed in arbitrary units (a.u.) and shown in pseudocolour, from dark (lowest) to white (highest). (B) Frequency map of the Ca2+ activity in the astrocyte during the 165 s period as in A. Activity is measured in individual pixels, expressed in mHz and color-coded from black (never active) to dark red (frequently active). Most of the activity is within the white boundaries and the most frequently active pixels are in defined small regions (arrowheads) of the primary and secondary processes (30 mHz), whereas pixels of the soma are less active (~10 mHz) (Volterra et al., 2014).

Free intracellular calcium ions were measured using the fluorescent calcium indicator FLUO-3/AM (Molecular probes, Eugene, OR, USA). Cells (4×10^4 cells/cm^2) were seeded in 24-well plates for 24 h to reach 60%–70%, and then treated for 24 h with As(III) (0.5 and 1 mg/l), or coexposed to As(III) (1 mg/l) and F (2.5, 5, and 10 mg/l). After treatment, supernatant was collected and combined with trypsinized cells. Pelleted samples were resuspended in 500 μl of FLUO-3/AM (4 μmol/l) and incubated at 37 °C for 30 min. After centrifugation, cells were washed with HBSS (Hank's Buffered Salt Solution, Sigma), made up to 400 μl with HBSS and analyzed by flow cytometry. The signal from FLUO-3/AM bound to Ca^{2+} was recorded using the Fl-1 channel (Rocha et al., 2011).

Fluo-4/AM was used as an intracellular free Ca^{2+} fluorescent probe to analyze $[Ca^{2+}]_i$ in Cd-exposed cerebral cortical neurons. In short, the harvested cells were incubated with Fluo-4/AM (5 μmol/L final concentration) for 30 min at 37°C in the dark, washed with PBS, and analyzed on a BD-FACS Aria flow cytometry. Intracellular $[Ca^{2+}]_i$ levels were represented by fluorescent intensity. Fluorescent intensity was recorded by excitation at 494 nm and emission at 516 nm. The data were analyzed by Cell Quest program (Becton Dickinson), and the mean fluorescence intensity was obtained by histogram statistics (Yuan et al., 2013).

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Event: 177: N/A, Mitochondrial dysfunction 1

Short Name: N/A, Mitochondrial dysfunction 1

Key Event Component

Process	Object	Action
	mitochondrion	functional change

AOPs Including This Key Event

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:326 - Thermal stress leading to population decline (3)	MolecularInitiatingEvent
Aop:325 - Thermal stress leading to population decline (2)	MolecularInitiatingEvent

AOP ID and Name	Event Type
Aop:324 - Thermal stress leading to population decline (1)	MolecularInitiatingEvent
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 heart failure 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

Stressors

Name
Uranium
Nanoparticles and Micrometer Particles
Cadmium

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Uranium

Shaki et al. (2012) found that uranyl acetate (UA) exposure in isolated rat kidney mitochondria decreased the ATP production levels and ATP/ADP ratio in a concentration-dependent manner, through inhibition of complexes II and III of the ETC. Both of these levels were significantly changed at UA concentrations of 100 µM and 200 µM. In addition, a concentration-dependent decrease in activity of complex II with exposure to uranium (U) was observed (Shaki et al., 2012). They also found that mitochondrial membrane potential damage and mitochondrial swelling significantly increased both time- and dose-dependently in the treated rat kidneys (Shaki et al., 2012). ATP/ADP ratios were also decreased significantly by treatment with 100 µM or more of uranium (Shaki et al., 2012). Mitochondrial outer membrane damage was significantly decreased by treatment with 200 µM of uranium (Shaki et al., 2012).

Shaki et al. (2013) also investigated the effects of uranium on rat kidneys. They found that mitochondrial permeability transition was also impacted by uranium treatment, causing increased mitochondrial swelling and increased disruption of energy homeostasis

(Shaki et al., 2013).

Hao et al. (2014) assessed the changes in mitochondrial potential in human kidney proximal tubular cells treated with uranium and found that the group treated with 500 µM of depleted uranium for 24 hours showed a significant decrease in mitochondrial membrane potential.

In their study of the effects of depleted uranium treatment on human embryonic kidney cells, Hao et al. (2016) found that ETHE1, a mitochondrial protein involved in mitochondrial homeostasis and mitochondrial diseases, had significant dose- and time-dependant decreases in gene expression when treated with 125 µM or more depleted uranium (DU) for 2 hours or more.

Nanoparticles and Micrometer Particles

Karlsson et al. (2009) conducted experiments to examine the effects of micrometer and nanoparticle treatments of copper and iron on human alveolar type-II epithelial cells. Their results showed that copper oxide micrometer and nanoparticle treatments were able to cause dose-dependant mitochondrial depolarization with doses as low as 5 µg/cm^2 (Karlsson et al., 2009). Iron(III) oxide nanoparticles and micrometer particles were both able to cause similar amounts of mitochondrial depolarization, along with iron (IV) oxide micrometer particles, however they were all much less toxic than copper oxide nanoparticles or micrometer particles (Karlsson et al., 2009).

The effects of gold nanoparticle (Au1.4MS) treatment on human cervical cancer cells were assessed by Pan et al. (2009), who found that the treated cells experienced a significant increase in permeability transition.

Huerta-García et al. (2014) studied the effects of titanium oxide nanoparticle treatment on glial tumor rat neuronal cells and cancerous human brain cells. Their results showed that in the treated rat and human cells there was a clear time-dependant increase in depolarization (Huerta-García et al., 2014). Both the human and rat cells showed time-dependant decreases in mitochondrial membrane potential. The TiO_2 nanoparticles were more toxic to the human cells than to the rat cells. The human cells showed a significant decrease in mitochondrial membrane potential as early as 2 hours post-treatment, while the rat cells did not show significant decrease until 6 hours post-treatment (Huerta-García et al., 2014).

Zhang et al. (2018) investigated the effects of copper nanoparticles on mitochondrial membrane potential in pig kidney cells and found that the treated cells showed a dose-dependant increase in the rate of mitochondrial membrane potential change from 40 µg/mL to 80 µg/mL when treated for 12 hours.

Cadmium

Belyaeva et al. (2012) studied the effects of cadmium treatment on rat kidney cells. In particular, they looked at different respiration rates in treated and untreated rat kidney cell lines. They found that resting respiration rates were significantly stimulated at 48 hours of treatment with 100 µM of cadmium, while uncoupled respiration was unaffected, and basal respiration was enhanced (Belyaeva et al., 2012). These changes in respiration imply that cadmium was capable of reducing the uncoupling efficiency of the cells at concentrations of 100 µM or higher.

Miccadei and Floridi (1993) studied changes in oxygen consumption in rat liver mitochondria which had been treated with cadmium. Their results showed that the treated rats showed a dose-dependant decrease in oxygen consumption which began with doses as low as 3 µM (Miccadei and Floridi, 1993).

Wang et al. (2009), found that, when applied together, lead and cadmium showed individual inhibition and additive effects of rat kidney mitochondrial COX gene expression.

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

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).

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 (O_2^-). 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 Ca^{2+} signaling and ROS production (reviewed Mei et al., 2013). Prolonged ER stress leads to release of Ca^{2+} at the MAM and increased Ca^{2+} uptake into the mitochondrial matrix, which induces Ca^{2+}-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 Ca^{2+}, increased mitochondrial uptake of Ca^{2+}, 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 Ca^{2+} flux which induce ER stress. Increased Ca^{2+} 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).

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 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 Heimburg et al., 2005).

2. Mitochondrial membrane potential (Δψm).

The mitochondrial membrane potential (Δψm) 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. The classical, and still most quantitative method uses a tetraphenylphosphonium ion (TPP+)-sensitive electrode on suspensions of isolated mitochondria. The Δψm can also be measured in live cells by fluorimetric methods. These are based on dyes which accumulate in mitochochondria because of Δψm. Frequently used are tetramethylrhodamineethylester (TMRE), tetramethylrhodaminemethyl ester (TMRM) (Petronilli et al., 1999) or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1). Mitochondria with intact membrane potential concentrate JC-1, so that it forms red fluorescent aggregates, whereas de-energized mitochondria cannot concentrate JC-1 and the dilute dye fluoresces green (Barrientos et al., 1999). Assays using TMRE or TMRM measure only at one wavelength (red fluorescence), and depending on the assay setup, de-energized mitochondria become either less fluorescent (loss of the dye) or more fluorescent (attenuated dye quenching).

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

Determination of ETS activity can be dene 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 [l - (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).

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 (GSSH) by the action of an NADPH dependent reductase (GSSH + NADPH + H+ à 2 GSH + NADP+). The ratio of GSH/GSSG is therefore a good indicator for

the cellular NADH+/NADPH ratio (i.e. the redox potential). GSH and GSSH 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 to ferrocytochrome 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 (H_2O_2) 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 H_2O_2 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 Schimadzou 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 accurancy
TMRE fluorescence Assay Measuring Mitochondrial permeability	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	1 µM cyclosporin	Short / easy

transition pore (mPTP) opening (Huser et al., 1998)	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.	A	Low accurancy
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 determinted 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 accurancy
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 accurancy
Aequorin-based bioluminescence assay Increase in mitochondrial Ca^{2+} influx (Pozzan & Rudolf, 2009)	Together with GFP, the aequorin moiety acts as Ca^{2+} sensor *in vivo*, which delivers emission energy to the GFP acceptor molecule in a BRET (Bioluminescence Resonance Energy Transfer) process; the Ca2+ can then be visualized with fluorescence microscopy.		Short / easy Low accurancy
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 accurancy
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 mono- clonal 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 accurancy
Membrane potential and cell viability – Flow Cytometry Measuring cytochrome c release	"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,		Short / easy Medium accurancy

(Kruidering et al., 1997)	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 *et al.*, 1993)"		

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Event: 1115: Increased, Reactive oxygen species

Short Name: Increased, Reactive oxygen species

Key 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: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 - ERa 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

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

ROS is a normal constituent found in all organisms.

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 O2- derived molecules that can be both free radicals (e.g. superoxide, hydroxyl, peroxyl, alcoxyl) 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).

How it is Measured or Detected

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

Yuan, Yan, et al., (2013) described ROS monitoring by using H_2-DCF-DA, a redox-sensitive fluorescent dye. Briefly, the harvested cells were incubated with H_2-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.

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Event: 1262: Apoptosis

Short Name: Apoptosis

Key Event Component

Process	Object	Action
apoptotic process		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:205 - AOP from chemical insult to cell death	AdverseOutcome
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:212 - Histone deacetylase inhibition leading to testicular atrophy	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	KeyEvent
Aop:439 - Activation of the AhR leading to metastatic breast cancer	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:393 - AOP for thyroid disorder caused by triphenyl phosphate via TRβ activation	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	KeyEvent
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:491 - Decrease, GLI1/2 target gene expression leads to orofacial clefting	KeyEvent

AOP ID and Name	Event Type
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:502 - Decrease, cholesterol synthesis leads to orofacial clefting	KeyEvent
Aop:441 - Ionizing radiation-induced DNA damage leads to microcephaly via apoptosis and premature cell differentiation	KeyEvent

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
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Caenorhabditis elegans	Caenorhabditis elegans	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

Apoptosis is induced in human prostate cancer cell lines (*Homo sapiens*) [Parajuli et al., 2014].

Apoptosis occurs in B6C3F1 mouse (*Mus musculus*) [Elmore, 2007].

Apoptosis occurs in Sprague-Dawley rat (*Rattus norvegicus*) [Elmore, 2007].

Apoptosis occurs in the nematode (*Caenorhabditis elegans*) [Elmore, 2007].

- Apoptosis occurs in breast cancer cells, human and mouse (Parton)

Key Event Description

Apoptosis, the process of programmed cell death, is characterized by distinct morphology with DNA fragmentation and energy dependency [Elmore, 2007]. Apoptosis, also called “physiological cell death”, is involved in cell turnover, physiological involution, and atrophy of various tissues and organs [Kerr et al., 1972]. The formation of apoptotic bodies involves marked condensation of

both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances [Kerr et al., 1972]. Apoptosis is characterized by DNA ladder and chromatin condensation. Several stimuli such as hypoxia, nucleotides deprivation, chemotherapeutical drugs, DNA damage, and mitotic spindle damage induce p53 activation, leading to p21 activation and cell cycle arrest [Pucci et al., 2000]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation of the NHDF cells [Glaser et al., 2003]. Considering that the acetylation of histone H4 was increased by the treatment of SAHA for 4 hrs, histone deacetylase inhibition may be involved in the inhibition of the cell proliferation [Glaser et al., 2003]. The impaired proliferation was observed in HDAC1$^{-/-}$ ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz et al., 2010]. An AOP focuses existes on p21 pathway leading to apoptosis, however, alternative pathways such as NF-kappaB signaling pathways may be involved in the apoptosis of spermatocytes [Wang et al., 2017].

Apoptosis is defined as a programmed cell death. A decrease in apoptosis or a resistance to cell death is noted is described as a hallmark of cancer by Hanahan et al. It is widely admitted as an essential step in tumor proliferation (Adams, Lowe). Apoptosis occurs after activation of a number of intrinsic and extrinsic signals which activate the protease caspase system which in turn activates the destruction of the cell.

The Bcl-2 is a protein family suppressing apoptosis by binding and inhibiting two proapoptotic proteins (Bax and Bak) and transferring them to the mitochondrial outer membrane. In the absence of inhibition by Bcl2, Bax and Bak destroy the mitochondrial membrane and releases proapoptotic signaling proteins, such as cytochrome *c which activated the caspase system.* An increased expression of these antiapoptotic proteins (Bcl-2, Bcl-x_L) *occurs in cancer (Hanahan, Adams, Lowe). Several others pathways such as the l*oss of TP53 tumor suppressor function, or the increase of survival signals (Igf1/2), or decrease of proapoptotic factors (Bax, Bim, Puma) can also increase tumor growth *(Hanahan, Juntilla).*

In breast cancer a decrease in apoptosis and a resistance to cell death has been described thoroughly, especially using a dysregulation of the Bcl2 system or TP53 (Parton, Williams, Shahbandi).

How it is Measured or Detected

Apoptosis is characterized by many morphological and biochemical changes such as homogenous condensation of chromatin to one side or the periphery of the nuclei, membrane blebbing and formation of apoptotic bodies with fragmented nuclei, DNA fragmentation, enzymatic activation of pro-caspases, or phosphatidylserine translocation that can be measured using electron and cytochemical optical microscopy, proteomic and genomic methods, and spectroscopic techniques [Archana et al., 2013; Martinez et al., 2010; Taatjes et al., 2008; Yasuhara et al., 2003].

DNA fragmentation can be quantified with comet assay using electrophoresis, where the tail length, head size, tail intensity, and head intensity of the comet are measured [Yasuhara et al., 2003].

The apoptosis is detected with the expression alteration of procaspases 7 and 3 by Western blotting using antibodies [Parajuli et al., 2014].

The apoptosis is measured with down-regulation of anti-apoptotic gene baculoviral inhibitor of apoptosis protein repeat containing 2 (BIRC2, or cIAP1) [Parajuli et al., 2014].

Apoptotic nucleosomes are detected using Cell Death Detection ELISA kit, which was calculated as absorbance subtraction at 405 nm and 490 nm [Parajuli et al., 2014].

Cleavage of PARP is detected with Western blotting [Parajuli et al., 2014].

Caspase-3 and caspase-9 activity is measured with the enzyme-catalyzed release of p-nitroanilide (pNA) and quantified at 405 nm [Wu et al., 2016].

Apoptosis is measured with Annexin V-FITC probes, and the relative percentage of Annexin V-FITC-positive/PI-negative cells is analyzed by flow cytometry [Wu et al., 2016].

Apoptosis is detected with the Terminal dUTP Nick End-Labeling (TUNEL) method to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks [Kressel and Groscurth, 1994].

For the detection of apoptosis, the testes are fixed in neutral buffered formalin and embedded in paraffin. Germ cell death is visualized in testis sections by Terminal dUTP Nick End-Labeling (TUNEL) staining method [Wade et al., 2008]. The incidence of TUNEL-positive cells is expressed as the number of positive cells per tubule examined for one entire testis section per animal [Wade et al., 2008]

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Event: 352: N/A, Neurodegeneration

Short Name: N/A, Neurodegeneration

Key Event Component

Process	Object	Action
neurodegeneration		increased

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	AdverseOutcome
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:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	AdverseOutcome
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:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Stressors

Name
Sars-CoV-2
Chemical
SARS-CoV
Virus

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
zebrafish	Danio rerio	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Mixed	High

The necrotic and apoptotic cell death pathways are quite well conserved throughout taxa (Blackstone and Green, 1999, Aravind et al., 2001). It has been widely suggested that apoptosis is also conserved in metazoans, although despite conservation of Bcl-2 proteins, APAF-1, and caspases there is no biochemical evidence of the existence of the mitochondrial pathway in either C. elegans or Drosophila apoptosis (Baum et al., 2007; Blackstone and Green, 1999).

Key Event Description

The term neurodegeneration is a combination of two words - "neuro," referring to nerve cells and "degeneration," referring to progressive damage. The term "neurodegeneration" can be applied to several conditions that result in the loss of nerve structure and function, and neuronal loss by necrosis and/or apoptosis

Neurodegeneration is a key aspect of a large number of diseases that come under the umbrella of “neurodegenerative diseases" including Huntington's, Alzheimer’s and Parkinson’s disease. All of these conditions lead to progressive brain damage and neurodegeneration.

Alzheimer's disease is characterised by loss of neurons and synapses in the cerebral cortex and certain subcortical regions, with gross atrophy of the affected regions; symptoms include memory loss.

Parkinson's disease (PD) results from the death of dopaminergic neurons in the midbrain substantia nigra pars compacta; symptoms include bradykinesia, rigidity, and resting tremor.

Several observations suggest correlative links between environmental exposure and neurodegenerative diseases, but only few suggest causative links:

Only an extremely small proportion (less than 5%) of neurodegenerative diseases are caused by genetic mutations (Narayan and Dragounov, 2017). The remainders are thought to be caused by the following:

- A build up of toxic proteins in the brain (Evin et al., 2006)
- A loss of mitochondrial function that leads to the oxidative stress and creation of neurotoxic molecules that trigger cell death (apoptotic, necrotic or autophagy) (Cobley et al., 2018)
- Changes in the levels and activities of neurotrophic factors (Kazim and Iqbal, 2016; Machado et al., 2016; Rodriguez et al., 2014)
- Variations in the activity of neural networks (Greicius and Kimmel, 2012)

Protein aggregation: the correlation between neurodegenerative disease and protein aggregation in the brain has long been recognised, but a causal relationship has not been unequivocally established (Lansbury et al., 2006; Kumar et al., 2016). The dynamic nature of protein aggregation mean that, despite progress in understanding its mechanisms, its relationship to disease is difficult to determine in the laboratory.

Nevertheless, drug candidates that inhibit aggregation are now being tested in the clinic. These have the potential to slow the progression of Alzheimer's disease, Parkinson's disease and related disorders and could, if administered pre-symptomatically, drastically reduce the incidence of these diseases.

Loss of mitochondrial function: many lines of evidence suggest that mitochondria have a central role in neurodegenerative diseases (Lin and Beal, 2006). Mitochondria are critical regulators of cell death, a key feature of neurodegeneration. Dysfunction of mitochondria induces oxidative stress, production of free radicals, calcium overload, and mutations in mitochondrial DNA that contribute to neurodegenerative diseases. In all major examples of these diseases there is strong evidence that mitochondrial dysfunction occurs early and acts causally in disease pathogenesis. Moreover, an impressive number of disease- specific proteins interact with mitochondria. Thus, therapies targeting basic mitochondrial processes, such as energy metabolism or free-radical generation, or specific interactions of disease-related proteins with mitochondria, hold great promise.

Decreased level of neurotrophic factors: decreased levels and activities of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), have been described in a number of neurodegenerative disorders, including Huntington's disease, Alzheimer disease and Parkinson disease (Zuccato and Cattaneo, 2009). These studies have led to the development of experimental strategies aimed at increasing BDNF levels in the brains of animals that have been genetically altered to mimic the

aforementioned human diseases, with a view to ultimately influencing the clinical treatment of these conditions. Therefore BDNF treatment is being considered as a beneficial and feasible therapeutic approach in the clinic.

Variations in the activity of neural networks: Patients with various neurodegenerative disorders show remarkable fluctuations in neurological functions, even during the same day (Palop et al., 2006). These fluctuations cannot be caused by sudden loss or gain of nerve cells. Instead, it is likely that they reflect variations in the activity of neural networks and, perhaps, chronic intoxication by abnormal proteins that the brain is only temporarily able to overcome.

Neurodegeneration in relation to COVID19

SARS-CoV-2 patients present elevated plasma levels of neurofilament light chain protein (NfL), which is a well-known biochemical indicator of neuronal injury (Kanberg et al., 2020). Postmortem brain autopsies demonstrate virus invasion to different brain regions, including the hypothalamus and olfactory bulb, accompanied by neural death and demyelination (Archie and Cucullo 2020; Heneka et al. 2020).

Autopsy results of patients with SARS showed ischemic neuronal damage and demyelination; viral RNA was detected in brain tissue, particularly accumulating in and around the hippocampus (Gu et al. 2005).

Brain magnetic resonance imaging (MRI) investigations in SARS-CoV-2 patients show multifocal hyperintense white matter lesions and cortical signal abnormalities (particularly in the medial temporal lobe) on fluid-attenuated inversion recovery (FLAIR), along with intracerebral hemorrhagic and microhemorrhagic lesions, and leptomeningeal enhancement (Kandemirli et al. 2020; Kremer et al. 2020; Mohammadi et al., 2020).

Moreover, eight COVID-19 patients with signs of encephalopathy had anti–SARS-CoV-2 antibodies in their CSF, and 4 patients had CSF positive for 14-3-3-protein suggesting ongoing neurodegeneration (Alexopoulos et al. 2020).

How it is Measured or Detected

The assays for measurements of necrotic or apoptotic cell death are described in the Key Event: Cell injury/Cell death

Recent neuropathological studies have shown that Fluoro-Jade, an anionic fluorescent dye, is a good marker of degenerating neurons. Fluoro-Jade and Fluoro-Jade B were found to stain all degenerating neurons, regardless of specific insult or mechanism of cell death (Schmued et al., 2005). More recently, Fluoro-Jade C was shown to be highly resistant to fading and compatible with virtually all histological processing and staining protocols (Schmued et al., 2005). In addition, Fluoro-Jade C is a good tool for detecting acutely and chronically degenerating neurons (Ehara and Ueda, 2009).

Regulatory Significance of the AO

Currently the four available OECD Test Guidelines (TGs) for neurotoxicity testing are entirely based on in vivo neurotoxicity studies: (1)Delayed Neurotoxicity of Organophosphorus Substances Following Acute Exposure (TG 418); (2) Delayed Neurotoxicity of Organophosphorus Substances: 28-day Repeated Dose Study (TG 419); (3) Neurotoxicity Study in Rodents (TG 424) involves daily oral dosing of rats for acute, subchronic, or chronic assessments (28 days, 90 days, or one year or longer); (4) Developmental Neurotoxicity (DNT) Study (TG 426) evaluates in utero and early postnatal effects by daily dosing of at least 60 pregnant rats from implantation through lactation. One of the endpoints required by all four of these OECD TGs is evaluation of neurodegeneration that, so far, is performed through in vivo neuropathological and histological studies. Therefore, neurodegeneration described in this AOP as a key event, has a regulatory relevance and could be performed using in vitro assays that allow a reliable evaluation of neurodegeneration using a large range of existing assays, specific for apoptosis, necrosis and autophagy (see also KE Cell injury/Cell death).

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List of Adverse Outcomes in this AOP

Event: 341: Impairment, Learning and memory

Short Name: Impairment, Learning and memory

Key Event Component

Process	Object	Action
learning		decreased
memory		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	AdverseOutcome
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates	AdverseOutcome

neuronal cell death, contributing to learning and memory impairment.

AOP ID and Name	Event Type
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	AdverseOutcome
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:87 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KeyEvent
Aop:88 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure via abnormal role change within caste	KeyEvent
Aop:89 - Nicotinic acetylcholine receptor activation followed by desensitization contributes to abnormal foraging and directly leads to colony loss/failure	KeyEvent
Aop:90 - Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2	KeyEvent
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	AdverseOutcome
Aop:99 - Histamine (H2) receptor antagonism leading to reduced survival	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	AdverseOutcome
Aop:442 - Binding to voltage gate sodium channels during development leads to cognitive impairment	AdverseOutcome
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	AdverseOutcome
Aop:483 - Deposition of Energy Leading to Learning and Memory Impairment	AdverseOutcome
Aop:490 - Co-activation of IP3R and RyR leads to socio-economic burden through reduced IQ and non-cholinergic mechanisms	AdverseOutcome
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	AdverseOutcome
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	AdverseOutcome
Aop:520 - Retinoic acid receptor agonism during neurodevelopment leading to impaired learning and memory	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
fruit fly	Drosophila melanogaster	High	NCBI
zebrafish	Danio rerio	High	NCBI
gastropods	Physa heterostropha	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Mixed	High

Basic forms of learning behavior such as habituation have been found in many taxa from worms to humans (Alexander, 1990). More complex cognitive processes such as executive function likely reside only in higher mammalian species such as non-human primates and humans. Recently, larval zebrafish has also been suggested as a model for the study of learning and memory (Roberts et al., 2013).

Life stage applicability: This key event is applicable to various life stages such as during brain development and maturity (Hladik & Tapio, 2016).

Sex applicability: This key event is not sex specific (Cekanaviciute et al., 2018), although sex-dependent cognitive outcomes have been recently ; Parihar et al., 2020).

Evidence for perturbation by a prototypic stressor: Current literature provides ample evidence of impaired learning and memory being induced by ionizing radiation (Cekanaviciute et al., 2018; Hladik & Tapio, 2016).

Key Event Description

Learning can be defined as the process by which new information is acquired to establish knowledge by systematic study or by trial and error (Ono, 2009). Two types of learning are considered in neurobehavioral studies: a) associative learning and b) non-associative learning. Associative learning is based on making associations between different events. In associative learning, a subject learns the relationship among two different stimuli or between the stimulus and the subject's behaviour. On the other hand, non-associative learning can be defined as an alteration in the behavioural response that occurs over time in response to a single type of stimulus. Habituation and sensitization are some examples of non-associative learning.

The memory formation requires acquisition, retention and retrieval of information in the brain, which is characterised by the non-conscious recall of information (Ono, 2009). There are three main categories of memory, including sensory memory, short-term or working memory (up to a few hours) and long-term memory (up to several days or even much longer).

Learning and memory depend upon the coordinated action of different brain regions and neurotransmitter systems constituting functionally integrated neural networks (D'Hooge and DeDeyn, 2001). Among the many brain areas engaged in the acquisition of, or retrieval of, a learned event, the hippocampal-based memory systems have received the most study. For example, the hippocampus has been shown to be critical for spatial-temporal memory, visio-spatial memory, verbal and narrative memory, and episodic and autobiographical memory (Burgess et al., 2000; Vorhees and Williams, 2014). However, there is substantial evidence that fundamental learning and memory functions are not mediated by the hippocampus alone but require a network that includes, in addition to the hippocampus, anterior thalamic nuclei, mammillary bodies cortex, cerebellum and basal ganglia (Aggleton and Brown, 1999; Doya, 2000; Mitchell et al., 2002, Toscano and Guilarte, 2005; Gilbert et al., 2006, 2016). Thus, damage to variety of brain structures can potentially lead to impairment of learning and memory. The main learning areas and pathways are similar in rodents and primates, including man (Eichenbaum, 2000; Stanton and Spear, 1990).While the prefrontal cortex and frontostriatal neuronal circuits have been identified as the primary sites of higher-order cognition in vertebrates, invertebrates utilize paired mushroom bodies, shown to contain ~300,000 neurons in honey bees (Menzel, 2012; Puig et al., 2014).

For the purposes of this KE (AO), impaired learning and memory is defined as an organism's inability to establish new associative or non-associative relationships, or sensory, short-term or long-term memories which can be measured using different behavioural tests described below.

How it is Measured or Detected

In laboratory animals: in rodents, a variety of tests of learning and memory have been used to probe the integrity of hippocampal function. These include tests of spatial learning like the radial arm maze (RAM), the Barnes maze, Hebb-Williams maze, passive avoidance and Spontaneous alternation and most commonly, the Morris water maze (MWM). Test of novelty such as novel object recognition, and fear based context learning are also sensitive to hippocampal disruption. Finally, trace fear conditioning which incorporates a temporal component upon traditional amygdala-based fear learning engages the hippocampus. A brief description of these tasks follows.

1) RAM, Barnes, MWM, Hebb-Williams maze are examples of spatial tasks, animals are required to learn the location of a food reward (RAM); an escape hole to enter a preferred dark tunnel from a brightly lit open field area (Barnes maze), or a hidden platform submerged below the surface of the water in a large tank of water (MWM) (Vorhees and Williams, 2014). The Hebb-Williams maze measures an animal's problem solving abilities by providing no spatial cues to find the target (Pritchett & Mulder, 2004).

2) Novel Object recognition. This is a simpler task that can be used to probe recognition memory. Two objects are presented to animal in an open field on trial 1, and these are explored. On trial 2, one object is replaced with a novel object and time spent interacting with the novel object is taken evidence of memory retention – I have seen one of these objects before, but not this one

(Cohen and Stackman, 2015).

3) Contextual Fear conditioning is a hippocampal based learning task in which animals are placed in a novel environment and allowed to explore for several minutes before delivery of an aversive stimulus, typically a mild foot shock. Upon reintroduction to this same environment in the future (typically 24-48 hours after original training), animals will limit their exploration, the context of this chamber being associated with an aversive event. The degree of suppression of activity after training is taken as evidence of retention, i.e., memory (Curzon et al., 2009).

4) Trace fear conditioning. Standard fear conditioning paradigms require animals to make an association between a neutral conditioning stimulus (CS, a light or a tone) and an aversive stimulus (US, a footshock). The unconditioned response (CR) that is elicited upon delivery of the footshock US is freezing behavior. With repetition of CS/US delivery, the previously neutral stimulus comes to elicit the freezing response. This type of learning is dependent on the amygdala, a brain region associated with, but distinct from the hippocampus. Introducing a brief delay between presentation of the neutral CS and the aversive US, a trace period, requires the engagement of the amygdala and the hippocampus (Shors et al., 2001).

5) Operant Responding. Performance on operant responding reflects the cortex' ability to organize processes (Rabin et al., 2002).

In humans: A variety of standardized learning and memory tests have been developed for human neuropsychological testing, including children (Rohlman et al., 2008). These include episodic autobiographical memory, perceptual motor tests, short and long term memory tests, working memory tasks, word pair recognition memory; object location recognition memory. Some have been incorporated in general tests of intelligence (IQ) such as the Wechsler Adult Intelligence Scale (WAIS) and the Wechsler. Modifications have been made and norms developed for incorporating of tests of learning and memory in children. Examples of some of these tests include:

1) Rey Osterieth Complex Figure test (RCFT) which probes a variety of functions including as visuospatial abilities, memory, attention, planning, and working memory (Shin et al., 2006).

2) Children's Auditory Verbal Learning Test (CAVLT) is a free recall of presented word lists that yields measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak 1994; Talley, 1986).

3) Continuous Visual Memory Test (CVMT) measures visual learning and memory. It is a free recall of presented pictures/objects rather than words but that yields similar measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak, 1984; 1994).

4) Story Recall from Wechsler Memory Scale (WMS) Logical Memory Test Battery, a standardized neurospychological test designed to measure memory functions (Lezak, 1994; Talley, 1986).

5) Autobiographical memory (AM) is the recollection of specific personal events in a multifaceted higher order cognitive process. It includes episodic memory- remembering of past events specific in time and place, in contrast to semantic autobiographical memory is the recollection of personal facts, traits, and general knowledge. Episodic AM is associated with greater activation of the hippocampus and a later and more gradual developmental trajectory. Absence of episodic memory in early life (infantile amnesia) is thought to reflect immature hippocampal function (Herold et al., 2015; Fivush, 2011).

6) Staged Autobiographical Memory Task. In this version of the AM test, children participate in a staged event involving a tour of the hospital, perform a series of tasks (counting footprints in the hall, identifying objects in wall display, buy lunch, watched a video). It is designed to contain unique event happenings, place, time, visual/sensory/perceptual details. Four to five months later, interviews are conducted using Children's Autobiographical Interview and scored according to standardized scheme (Willoughby et al., 2014).

7) Attentional set-shifting (ATSET) task. Measures the ability to relearn cues over various schedules of reinforcement (Heisler et al., 2015).

8. Comprehensive developmental inventory for infants and toddlers (CDIIT). The CDIIT was designed and standardized in 1996, and it measures the global, cognitive, language, motor, gross motor, fine motor, social, self-help and behavioral developmental status of children from 3 to 71 months old (Wang et al., 1998).

In Honey Bees: For over 50 years an assay for evaluating olfactory conditioning of the proboscis extension reflex (PER) has been used as a reliable method for evaluating appetitive learning and memory in honey bees (Guirfa and Sandoz, 2012; LaLone et al., 2017). These experiments pair a conditioned stimulus (e.g., an odor) with an unconditioned stimulus (e.g., sucrose) provided immediately afterward, which elicits the proboscis extension (Menzel, 2012). After conditioning, the odor alone will lead to the conditioned PER. This methodology has aided in the elucidation of five types of olfactory memory phases in honey bee, which include early short-term memory, late short-term memory, mid-term memory, early long-term memory, and late long-term memory (Guirfa and Sandoz, 2012). These phases are dependent on the type of conditioned stimulus, the intensity of the unconditioned stimulus, the number of conditioning trials, and the time between trials. Where formation of short-term memory occurs minutes after conditioning and decays within minutes, memory consolidation or stabilization of a memory trace after initial acquisition leads to mid-term memory, which lasts 1 d and is characterized by activity of the cAMP-dependent PKA (Guirfa and Sandoz, 2012). Multiple conditioning trials increase the duration of the memory after learning and coincide with increased Ca2+-calmodulin-dependent PKC activity (Guirfa and Sandoz, 2012). Early long-term memory, where a conditioned response can be evoked days to weeks after conditioning requires translation of existing mRNA, whereas late long-term memory requires de novo gene transcription and can last for weeks (Guirfa andSandoz, 2012)."

Regulatory Significance of the AO

A prime example of impairments in learning and memory as the adverse outcome for regulatory action is developmental lead exposure and IQ function in children (Bellinger, 2012). Most methods are well established in the published literature and many have been engaged to evaluate the effects of developmental thyroid disruption. The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD TG 426) as well as OECD TG 443 (OECD, 2018) both require testing of learning and memory (USEPA, 1998; OECD, 2007) advising to use the following tests passive avoidance, delayed-matching-to-position for the adult rat and for the infant rat, olfactory conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze, T-maze, and acquisition and retention of schedule-controlled behaviour. These DNT Guidelines have been deemed valid to identify developmental neurotoxicity and adverse neurodevelopmental outcomes (Makris et al., 2009).

Also, in the frame of the OECD GD 43 (2008) on reproductive toxicity, learning and memory testing may have potential to be applied in the context of developmental neurotoxicity studies. However, many of the learning and memory tasks used in guideline studies may not readily detect subtle impairments in cognitive function associated with modest degrees of developmental thyroid disruption (Gilbert et al., 2012).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2942: Activation of MEK, ERK1/2 leads to Increase, intracellular calcium

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	adjacent	Not Specified	Not Specified
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	Moderate
1 to < 3 months	Moderate
Pregnancy	Moderate

Sex Applicability

Sex	Evidence
Female	Moderate
Mixed	Moderate

Key Event Relationship Description

Astrocytes are networked together by a series of gap junctions permitting to propagate Ca^{2+} waves through the linked network (Lobsiger and Cleveland 2007), and Ca2+-mediated intercellular communication is a mechanism by which astrocytes communicate with each other and modulate the activity of adjacent cells (Verderio et al., 2001). Metal mixture (MM) induced alteration in astrocyte morphology may influence $[Ca^{2+}]i$ (Barres et al., 1989); in contrast, an increase in $[Ca^{2+}]_i$ may also play a key role in altering astrocyte cytoskeleton, affecting the glia-neuron interaction (Shelton et al., 2000).

Inhibition of GFAP immunoreactivity by MM in developing brain appears to be caused by astrocyte apoptosis. In primary cultures of astrocytes, our data show that MM synergistically induced apoptosis (Rai and others 2010). This was manifested by the activation of MEK/ERK, followed by the activation of JNK pathways, which then enhanced intracellular Ca2+ levels and subsequently ROS generation.

Evidence Supporting this KER

Empirical Evidence

We treated the astrocytes with a metal-mixture (MM) of arsenic, cadmium, and lead and observed that the MM triggered $[Ca^{2+}]_i$ release (Rai and others 2010). The $[Ca2+]_i$ release reached its peak after 30 min of MM treatment. Similarly, MM triggered ROS generation, and the ROS generation reached its peak after 1 h of MM treatment. To investigate whether the $[Ca^{2+}]_i$ release was

ROS, ERK1/2, or JNK1/2 –dependent, we incubated the MM-treated astrocytes with an antioxidant (a-tocopherol, 200 lg/ml), PD98059 (10lM), or SP600125 (10lM). a-Tocopherol itself was nontoxic. We observed that PD98059 (10lM) or SP600125 (10lM) suppressed $[Ca^{2+}]_i$ release, but a-tocopherol (200 lg/ml) did not. This suggested that $[Ca^{2+}]_i$ release in MM-treated astrocytes was ERK1/2 and JNK1/2 dependent (Rai and others 2010).

Yael and Breitbart (2015) demonstrated for the first time that mouse sperm ERK1/2 is activated upon ZP addition, and that ERK1/2 mediates the elevation of intracellular Ca2+ in the sperm cell prior to the occurrence of the acrosome reaction. The fact that the acrosome reaction, induced by the Ca^{2+}-ionophore A23187, was not inhibited by U0126 suggests that ERK1/2 mediates the acrosome reaction by activating Ca2+ transport into the cell. Direct determination of intracellular $[Ca^{2+}]$ revealed that Ca2+ influx induced by EGF or ZP was completely blocked by U0126. Thus, it has been established that the increase in ERK1/2 phosphorylation/activation in response to ZP or by activation of the EGF receptor (EGFR) by EGF, is a key event for intracellular Ca^{2+} elevation and the subsequent occurrence of the acrosome reaction (Jaldety et al., 2015).

To examine the relationship between Ca^{2+} and Erk1/2 signaling, Levin and Borodinsky (2022) inhibited Mek1/2 with PD0325901 and found that this prevents the injury-induced increase in Ca^{2+} activity in cells lateral to the axial musculature across the entire 800 µm-wide region measured. This suggests that injury-induced Erk1/2 activation recruits Ca^{2+} activity to promote regeneration of the larval tail. Consistent with recruitment of Ca^{2+} activity across a wide region of tail, activated Erk1/2 is also present in at least the posterior 800 µm of stump (Levin et al., 2022). However, unlike Ca^{2+} activity, Erk1/2 signaling at 20 mpa is activated in a gradient. This could mean that even the lowest level of Erk1/2 signal measured in 800 µm of amputated tail is sufficient to induce the Ca^{2+} response, or that a signal is propagated anteriorly from the cells adjacent to the amputation where injury induces high Erk1/2 activation (Levin et al., 2022).

Quantitative Understanding of the Linkage

Time-scale

Exposures were conducted for 2 min, 5 min, 10 min, 30 min, 1 h, 2 h, and 24 h. The $[Ca2+]_i$ release reached its peak after 30 min of MM treatment (Rai and others 2010).

Known Feedforward/Feedback loops influencing this KER

The activity of many protein kinases is modulated by Ca2+ and/or Ca2+/calmodulin either directly (PKC, CaM kinase II) or indirectly (PKA via stimulation of adenylyl cyclase and phosphodiesterase by Ca2+/calmodulin) (Kern et al., 1995). Therefore, the effects of Ca2+ and protein kinases on cytoskeletal proteins and neurite initiation are likely to be mediated, at least in part, by changes in protein phosphorylation (Kern et al., 1995).

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Relationship: 3140: Increase, intracellular calcium leads to N/A, Mitochondrial dysfunction 1

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Key Event Relationship Description

One of the better characterized apoptotic cascade pathways has mitochondrial dysfunction as its initiator. Mitochondrial dysfunction initiated by the opening of the mitochondrial transition pore leads to mitochondrial depolarization, release of cytochrome C, activation of a variety of caspases and cleavage of downstream death effector proteins, and ultimately results in apoptotic cell death. While a variety of stimuli can trigger opening of the mitochondrial transition pore and cause apoptosis, a sustained intracellular increase in Ca2+ is one of the better-known triggers (Mattson 2000).

Evidence Supporting this KER

Biological Plausibility

Intracellular calcium overload may be related to the mitochondrial dysfunction (Yuan et al., 2013). Mitochondria are vital organelles for cellular metabolism and bioenergetics, but they are also key regulators of cell death (Fantin and Leder 2006). Since mitochondria are the major site of ATP production and mitochondrial ΔΨ is the driving force of ATP synthesis, a breakdown in the mitochondrial ΔΨ could lead to a fall in the ATP levels (Chakraborti et al., 1999). The resulting reduction in cellular ATP levels can disrupt ionic homeostasis which can cause an increase in $[Ca^{2+}]_i$ and subsequent cellular apoptosis/necrosis (Grammatopoulos et al., 2004). Notably, in many (if not all) paradigms of apoptosis, ΔΨm represents the point of no return in the cascade of events that ultimately leads to cell death (Kroemer et al., 2007).

The effector phase of apoptosis involves increased mitochondrial Ca^{2+} and oxyradical levels, the formation of permeability transition pores (PTP) in the mitochondrial membrane, and release of cytochrome c into the cytosol (Mattson 2000).

The increase of free radicals and Ca^{2+} levels associated to Cd exposure may induce mitochondrial disruption (Fern et al., 1996).

Intracellular calcium homeostasis is very important in maintaining the normal function of the cell, in that variations in the concentration of calcium in cells can determine cell survival or death. For example, a high $[Ca^{2+}]i$ can cause disruption of mitochondrial Ca^{2+} equilibrium, which results in reactive oxygen species (ROS) formation due to the stimulation of electron flux along the electron transport chain (ETC) (Chacon and Acosta 1991). Under oxidative stress, mitochondrial Ca^{2+} accumulation can switch from a physiologically beneficial process to a cell death signal (Ermak and Davies 2002).

Empirical Evidence

Yuan, Yan, et al. 2013 found that BAPTA-AM significantly blocked disruption of Δψm in cells exposed to Cd (5, 10 and 20 μM) for 12 h. Furthermore, cleavage of caspase-9, caspase-3 and PARP were significantly attenuated by BAPTA-AM, which was in agreement with thier observation that BAPTA-AM profoundly prevented Cd-induced apoptosis and cell death of cerebral cortical neurons. However, increased Bax and decreased Bcl-2 levels were not blocked by BAPTA-AM . These data suggest that calcium-mediated mitochondria-caspase b is involved in Cd-induced apoptosis. Moreover, thier results collectively suggested that Cd-induced apoptosis of cerebral cortical neurons occurs through a calcium-mitochondria signaling pathway (Yuan et al., 2013).

Yuan, Yan, et al. 2013 also noted that reduced expression of Bcl-2 increases the expression of Bax, which results in an overload of Ca^{2+} in the mitochondria and promotes the opening of permeability transition pores causing mitochondria to swell, with their outer membranes collapsing and exiting into the cytoplasm, which would consequently trigger apoptosis.

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Relationship: 3141: N/A, Mitochondrial dysfunction 1 leads to Increased, Reactive oxygen species

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Mus musculus	Mus musculus	Moderate	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Key Event Relationship Description

Mitochondria play a role in stress responses and can produce ROS when damaged. Mitochondria are indeed a major source of ROS (Yuan et al., 2013). ROS production is related to the level of ETC (Fleury et al., 2002); it is elevated when electron transport is reduced, which occurs in pathological situations (Wallace 2005).

Evidence Supporting this KER

Biological Plausibility

A phenotype that is commonly associated with mitochondrial dysfunction, and in fact with many age-related diseases, is the accumulation of damage attributable to the buildup of reactive oxygen species (Leadsham et al., 2013). Indeed, a vicious cycle of decline in which ROS arising from the mitochondrial electron transport chain (ETC) leads to the damage to mitochondrial DNA and a resultant increase in radical production provides the cornerstone of the much scrutinized free radical theory of aging (Harman 1956). However, ROS also serve as important signaling molecules that can promote longevity in C. elegans (Schulz et al., 2007)

and also in yeast (Mesquita et al., 2010).

Alterations in mitochondrial physiology could be involved in programmed cell death (PCD). First, reactive oxygen species (ROS) may participate as effector molecules in PCD (Hockenbery et al., 1993; Kane et al., 1993; Sandstrom et al., 1994).

Empirical Evidence

Lopez et al. (2006) showed that in cortical neurons, cadmium exposure induced cellular death, which was, in part, reversed by vitamin C, an antioxidant agent. The apoptosis produced by cadmium was reversed by vitamin C while the necrosis was not affected by this antioxidant molecule. It also appears that in the apoptotic mechanism mediated by cadmium, but not in the necrotic mechanisms, oxidative stress could be implicated. The ability of cadmium to induce oxidative stress in cortical neurons is aided by the induction of ROS by this cation. Cortical neurons treated with cadmium ions at concentrations between 1 and 100 µM, in either the absence or in the presence of serum in the treatment medium, generated ROS. The induction of ROS in these cells type could be mediated by mitochondria alterations because cadmium produces a breakdown of the mitochondrial membrane potential. The decreases in ATP levels and in the mitochondria membrane potential began at 10 and 50 µM cadmium ion, respectively, while the ROS formation was detected at lower doses (100 nM or 1 µM). These results likely indicate that ROS formation occurs or it is detectable before the toxic events on mitochondrial function that lead to the breakdown in mitochondrial potentials.

Zamzami et al. (1995) concluded that at a final level, the shrinkage of $\Delta\Psi_m^{low}HE^+$ cells is selectively inhibited by substances that suppress mitochondrial ROS generation (rotenone, ruthenium red), as well as by antioxidants such as the vitamin E derivative trolox, alone or incombination with L-ascorbate, or the radical scavenger N-t-butyl-alpha-phenylnitrone. This observation confirms that ROS are PCD effector molecules. In synthesis, these data indicate that $\Delta\Psi_m$ reduction and enhanced mitochondrial ROS generation indeed represent two clearly distinct phases of the preapoptotic process. Only after $\Delta\Psi_m$ has dropped are ROS generated and do they participate in the perturbation of mitochondrial membranes, as well as in later manifestations of PCD such as cell shrinkage.

Zamazim et al. (1995) went on to state that reduction in $\Delta\Psi_m$ and subsequent KOS hyperproduction are observed in several in vitro models of physiological PCD, i.e., models in which nontoxic agents were used to induce PCD in susceptible target cells: TNF-a in U937 cells and anti-IgM in WEHI 231 pre-B cells, as well as CD3 cross-linking in T cell hybridomas. Ceramide, a second messenger involved in the mediation of some PCD types (Obeid et al., 1993; Haimovitz-Friedman et al., 1994), also causes these effects. In all of these systems, alterations in mitochondrial function precede DNA fragmentation and nuclear DNA loss. Thus, it appears that mitochondrial derangement is a constant feature of PCD occurring independently of the PCD-inducing stimulus.

Zhang et al. (2004) reported that Mn^{2+} exposure inhibited the complexes I–IV compared to the control. The inhibition of the respiratory activity by Mn^{2+} is accompanied by a substantial increase of ROS production rate. They went on to report that NAC, GSH and vitamin C are effective in the prevention of Mn^{2+}-induced ROS production and decreases of complexes I–IV activity in isolated mitochondria. Preventive effects of NAC and GSH reveal that cellular GSH are crucial for protection against Mn^{2+}-induced toxicity.

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Relationship: 2966: Increased, Reactive oxygen species leads to Apoptosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Key Event Relationship Description

ROS generation in normal cells, including neurons, occurs within homeostatic control. When ROS levels exceed the antioxidant capacity of a cell, a deleterious condition known as oxidative stress occurs (Klein and Ackerman 2003). Unchecked, excessive ROS can lead to the destruction of cellular components including lipids, protein, and DNA, and ultimately cell death via apoptosis or necrosis (Kannan and Jain 2000).

Evidence Supporting this KER

Biological Plausibility

Reactive oxygen species (ROS) can be derived from exogenous sources or produced in vivo; these include the superoxide anion (O 2), the hydroxyl radical (OH), and hydrogen peroxide (H 2O2). ROS at low levels participate in cell signaling while higher ROS concentrations are deleterious due to the oxidation of proteins, lipids, and DNA. Additionally, persistent ROS production compromises the cellular antioxidant defense systems and results in oxidative stress and apoptosis (337). ROS can initiate apoptosis via the mitochondrial and death receptor pathways. In the former, ROS have been shown to induce loss of the m, release of mitochondrial pro-apoptotic proteins, and activation of caspase 3 (49).

ROS signaling has been shown to mediate cytokine-induced apoptosis (Okouchi et al., 2007). TNF is a pro-inflammatory cytokine produced by macrophages and is the most studied cytokine in apoptosis and the pathophysiology of various diseases, including neurodegenerative disorders (Jackson et al., 1999). Mechanistically, the binding of TNF to its receptor activates the NF-B and JNK signaling pathways believed to be mediated by ROS (Okouchi et al., 2007). A role for ROS has also been implicated in death receptor-mediated apoptosis induced by apoptosis signal-regulating kinase 1 (ASK1), an ubquitiously expressed MAP kinase kinase kinase (MAPKKK), that activates JNK and p38 MAP kinase pathways (Okouchi et al., 2007).

Empirical Evidence

Free radical scavenger or antioxidant N-acetyl-L-cysteine, a thiol-containing compound, has been shown to directly reduce the levels of ROS (Aruoma et al., 1989; Kim and Sharma 2004; Poliandri et al., 2003). To confirm that Cd-induced neuronal apoptosis is

indeed due to its induction of ROS generation, PC12 and SH-SY5Y cells were pretreated with NAC (5mM) for 1h, and then exposed to Cd (10 and 20μM) for 24h (Long et al., 2008). Chen et al. (2008) found that NAC dramatically blocked Cd-induced ROS generation in PC12 cells and SH-SY5Y cells. In addition, to further quantify the protective effect of NAC on Cd-induced apoptosis via blockage of ROS in a larger cell population, they performed annexin-V-FITC and propidium iodide staining followed by flow cytometry. They found NAC alone did not affect cell viability. However, it significantly blocked Cd-induced apoptosis.

Asit Rai et al. 2010 found that a metal mixture of arsenic, cadmium, and lead triggered ROS generation, reaching its peak after 1 hour of treatment. They next investigated whether ERK1/2, JNK1/2, $[Ca^{2+}]_i$ and ROS signaling resulted in apoptosis by reating the MM-treated astrocytes with α-tocopherol (200 μg/ml), PD98059 (10μM), BAPTA-AM (5μM), or SP600125 (10μM). They all suppressed apoptosis suggesting that activation of ERK1/2 and JNK1/2, followed by increased $[Ca^{2+}]_i$ and ROS generation, resulted in apoptosis in the MM-treated astrocytes.

When astrocytes were exposed to H2O2 for 30 min and then incubated without H2O2 for 1–5 days, cell toxicity including apoptosis was observed (Kazuhiro et al., 2004). Furthermore, the reperfusion injury induced by Ca^{2+} depletion or H2O2 exposure was exacerbated by the catalase inhibitor, 3-amino-1,2,4-triazole, and the GSH synthesis inhibitors, l-buthionine-S,R-sulfoximine and xanthine, while the injury was blocked by GSH, catalase and the iron chelators, 1,10-phenanthroline and deferoxamine (Takuma et al., 1999). These findings indicate that Ca^{2+} reperfusion-induced apoptosis is mediated by ROS production, especially by hydroxyl radical formation (Kazuhiro et al., 2004).

Exposure of cells to 5 M iAs significantly triggered the expression of ER stress-related molecules, including: the proteins and mRNAs expression of GRP 78, CHOP, XBP-1 in a time-dependent manner (for 6–24 h) as well as the degradation of full-length (55 kDa) caspase-12 (downstream ER stress molecule). However, GRP 94 was not affected by iAs treatment. These effects of iAs-induced ER stress protein responses could be reversed by pre-treatment with NAC. Furthermore, transfection of Neuro-2a cells with GRP 78- and CHOP-specific si-RNA, respectively, markedly reduced the protein expression levels of GRP 78 and CHOP in the cells treated with iAs and significantly attenuate the iAs-induced caspase-3, -7, and -12 activations. These results indicate that oxidative stress-mediated ER stress activation pathway is also involved in iAs-induced neuronal cell apoptosis (Tien-Hui, et al. 2014).

Recent studies have shown that ROS generation induced by toxic metals (including arsenic) causes neuronal apoptosis, which is closely associated with the progression of neurodegenerative diseases (Bharathi and Jagannathan 2006; Flora et al., 2009; Gharibzadeh 2008).

Okouchi et. al. (2007) found that peroxide-induced apoptosis in undifferentiated PC12 cells was mediated by an early loss of the cellular glutathione–glutathione disulfide (GSH/GSSG) redox balance that preceded an increase in Bax expression, mitochondrial-to-cytosol cytochrome c translocation, and activation of caspase 3 (Pias and Aw 2002; Pias and Aw 2002; Pias et al., 2003). Apoptosis was ameliorated by the overexpression of mitochondrial superoxide dismutase, MnSOD (SOD2), and by pretreatment of cells with the antioxidant, N-acetyl cysteine (NAC) [(23-25)].

As first demonstrated in mouse fibrosarcoma cells, TNF treatment disrupts mitochondrial electron transport and enhances ROS production (Schulze–Osthoff et al., 1992). Recent studies by Han et al. (2006) showed that modulation of the hepatocyte redox environment by ROS interfered with NF-B signaling in TNF-induced apoptosis. Notably, cell apoptosis occurred within a certain redox window in which mild redox imbalance inhibited NF-B activation, but not caspase activity (Okouchi et al., 2007).

Uncertainties and Inconsistencies

ROS and/or oxidative damage can activate gene transcription and transcribed genes may be implicated in either cell survival or cell death (Klein and Ackerman 2003).

The increase in reactive oxygen species at As(III) concentrations of 0.5 mg/l or more may play an apoptogenic role and/or be a consequence of events occurring during apoptosis (Rocha et al. 2011). It is generally reported that ROS cause an increase in $[Ca^{2+}]$i of various cell types, which might be one of the causes for the C17.2 cells to enter apoptosis (Rocha et al. 2011). According to Hool and Corry (2007), the redox control of Ca^{2+} transport is due to the fact that ROS can react with the thiol groups of protein that form part of the Ca^{2+} transporters or channels. Alternatively, mitochondrial matrix Ca^{2+} overload can lead to enhanced generation of reactive oxygen species, triggering the permeability transition pore, dissipation of transmembrane mithocondrial potential, and cytochrome c release (Brookes et al., 2004). In any case, the fact that treatment with various antioxidants (vitamin E, tocopherol, and quercetin) did not rescue the cells from death by apoptosis indicates that oxidative stress was not the main cause of the observed cell death (Rocha et al. 2011).

Superoxides and lipid peroxidation are increased during apoptosis induced by myriad stimuli (Bredesen 1995). However, generation of ROS may be a relatively late event, occurring after cells have embarked on a process of caspase activation (Green and Reed 1998). In this regard, attempts to study apoptosis under conditions of anoxia have demonstrated that at least some proapoptotic stimuli function in the absence or near absence of oxygen, which implies that ROSs are not the sine qua non of apoptosis (Jacobson and Raff 1995). However, ROSs can be generated under conditions of virtual anaerobiosis (Degli Esposti and McLennan 1998), and thus their role in apoptosis cannot be excluded solely on this basis (Green and Reed 1998).

Okouchi et. al. (2007) found that PC12 apoptosis can be initiated by GSH/GSSG redox imbalance alone independently of ROS generation (Pias et al., 2003), suggesting that a loss of cellular redox homeostasis is downstream of ROS signaling in neuronal cell apoptosis.

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Relationship: 2967: Apoptosis leads to N/A, Neurodegeneration

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Key Event Relationship Description

In the central nervous system (CNS), neuronal apoptosis is a physiological process that is an integral part of neurogenesis, and aberrant apoptosis has been implicated in the pathogenesis of neurodegeneration (Okouchi et al., 2007).

Evidence Supporting this KER

Biological Plausibility

During the development of the nervous system, an excessive number of neurons is produced. This massive overproduction of neurons is followed by a programmed demise of roughly one half of the originally produced cells (Okouchi et al., 2007). The precisely controlled process is referred to as naturally occurring neuronal death which is a highly conserved cellular mechanism in diverse organisms, ranging from invertebrate species such as the nematode (Okouchi et al., 2007), Caenorhabditis elegans, and insects, to nearly all of the studied vertebrate species (Mishima et al., 1999). Natural neuronal death is be lieved to mold the nervous system's cellular structure and function (Okouchi et al., 2007).

As axons extend, they also bifurcate with each branch forming of its own growth cone, a process that is also regulated by apoptosis (Chen et al., 2020). Under normal conditions, a low level of caspase maintains a balance between growth cone attraction and repulsion and inhibits axon extension; however, in PTSD, apoptosis is enhanced in key brain regions and caspase activation alters growth cone trajectory and dendritic pruning, leading to axon misguidance and dendrite degeneration. The combined outcome of these processes is the formation of fewer or incorrect synapses in PTSD that are defective in information transmission and cause abnormalities in memory and behavior (Chen et al., 2020).

Empirical Evidence

Rai, Nagendra Kumar, et al. (2013) concluded that the metal mixture arsenic, lead, and cadmium (a) induced dose-dependent modulation in the expression levels of myelin and axon proteins leading to hypo-myelination in cortex; (b) reduced axon area and

myelin density in O.N.; and (c) attenuated RGC-differentiation in retina. Apoptosis in the oligodendrocytes, axonal neurons and RGCs promoted the MM-mediated white matter damage.

Increased apoptosis in MBP and NF damage the white matter of CNS (Petzold et al., 2011; Pun et al., 2011). In consistence, Rai, Nagendra Kumar, et al. (2013) found that the impairment in postnatal oligodendrocytes and axons further increased cellular apoptosis in the brain and O.N. The neuroaxonal degeneration in the retina also involved a rise in apoptosis in Brn3b and NF. During development, Brn3b is crucial for RGC survival, and its apoptosis may affect the expression of several genes linked with axonal integrity and function (Pan, et al., 2005). Therefore, the increased MM-related apoptosis to axonal neurons in the retina could be the fallout of RGC damage (Rai et al., 2013). Altogether, the MM, in all probability, elevates the apoptosis-mediated pruning of the myelinating cells during CNS development (Rai et al., 2013).

In the context of an Alzheimer's disease brain; compelling evidence of apoptotic involvement comes from studies of Rohn et al., (2002) who demonstrated the activation of mitochondrial and receptor-mediated apoptotic pathways in AD hippocampal brain sections wherein active caspase 9 was co-localized with active caspase 8 (Okouchi et al., 2007). Moreover, the distribution of caspase-cleaved fragments of tau suggests that the activation of caspases preceded the formation of neurofibrillary tangles in brains of AD patients (Chiueh et al., 2000). In addition, the intracellular amyloid beta peptide 1-42 (A beta (1-42)) has been shown to induce human neuronal cell apoptosis through Bax activation that resulted in cytochrome c release and activation of caspase 6 (Zhang et al., 2002).

The participation of apoptosis in disease pathogenesis in humans is supported by the demonstration of caspases 1, 3, 8, and 9, and cytochrome c activation in the brains of Huntington Disease patients (Kiechle et al., 2022; Teng et al., 2006; Sanchez et al., 1999).

The involvement of hippocampal neuronal apoptosis in diabetic encephalopathy has been demonstrated in diabetic animal models (Li et al., 2005), and evidence of classical apoptosis was associated with decreased neuronal densities, and learning and cognitive deficits (Sima and Li 2005).

Cognitive impairment in BB/Wor rats is associated with evidence of classical apoptosis in the hippocampus, including DNA fragmentation, positive TUNEL staining, elevated Bax/Bcl-x ratio, increased caspase 3 activities and decreased neuronal densities (Li et al., 2002), common features in diabetic encephalopathy.

Notable among endogenous antioxidants, is estradiol, with proven effectiveness against beta-amyloid-induced neuronal apoptosis in in vitro models of AD and PD (Gandy 2003; Yao et al., 2007). Accelerated beta-amyloid plaque formation in animal models of AD is associated with brain estradiol deficiency (Gandy 2003). Estradiol mediates its effect by binding to the estrogen receptor, and targets a plethora of prosurvival cellular processes (Okouchi et al., 2007). These include neuronal expression of Bcl-2 members, upregulation of antioxidant proteins such as TRX, MnSOD, and nNOS, Akt signaling, and inhibition of transcriptional and apoptotic activity of the APPct complex (Yao et al., 2007; Bao et al., 2007; Chiueh et al., 2003; Koh et al., 2006). Melatonin is another naturally occurring neuroprotectant that decreases amyloid fibril formation (Pappolla et al., 1998) and attenuates neuronal apoptosis in in vitro and animal models of AD and PD (Chiueh et al., 2000; Deigner et al., 2000; Matsubara et al., 2003). Its neuroprotective effects appear to be the result of antioxidant and anti-amyloidogenic properties (Pappolla et al., 2002) and are independent of binding to membrane receptors (Okouchi et al., 2007).

Uncertainties and Inconsistencies

While the molecular mechanisms underlying neuronal apoptosis and diabetic encephalopathy remain unresolved, it appears that diabetes-associated perturbations in the insulin/IGF system and hyperglycemia may play prominent roles (Li and Sima 2004).

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Relationship: 1069: N/A, Neurodegeneration leads to Impairment, Learning and memory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	adjacent	High	
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	Moderate	NCBI
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Old Age	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Key Event Relationship Description

Animal models of neurodegenerative diseases, in particular Alzheimer's disease, contributed to the elucidation of the link between amyloid protein and tau hyperphosphorylation and cognitive deficits. Bilateral injections of amyloid-b peptide in the frontal cortex of rats leads to progressive decline in memory and neurodegeneration in hippocampus (for review see Eslamizade et al., 2016). Recent findings have shown that soluble forms of Ab rather than insoluble forms (fibrils and plaques) are associated with memory impairment in early stages of Alzheimer's disease (for review see Salgado-Puga and Pena-Ortega, 2015). Several lines of evidence suggest that the small oligomeric forms of Ab and tau may act synergistically to promote synaptic dysfunction in Alzheimer's disease (for review see Guerrerro-Minoz et al., 2015). Some reports proposed the concept of imbalance between production and clearance of Ab42 and related Ab peptides, as an initiating factor inducing hyperphosphorylation of tau and leading to neuritic dystrophy and synaptic dysfunction (for review see Selkoe and Hardy, 2016). Recent trials of three different antibodies against amyloid peptides have suggested a slowing of cognitive decline in post hoc analyses of mild Alzheimer subjects (for review see Selkoe and Hardy, 2016). Therefore cognitive deficits may be related to the level and extent of classical Alzheimer pathology landmarks, but it is also influenced by neurodegeneration (for review see Braskie and Thompson, 2013). Indeed decreased hippocampal volume due to widespread neurodegeneration and visualized by neuroimaging appears to be a significant predictor of memory decline (for review see Braskie and Thompson, 2016).

Evidence Supporting this KER

Biological Plausibility

It is well accepted that impairment of cell function or cell loss in hippocampus will interfere with memory processes, since the hippocampus plays a key role in memory (Barker and Warburton, 2011). In Alzheimer's disease, hippocampus and entorhinal cortex are affected early in the disease process and cognitive deficit is correlated with brain atrophy (for review Braskie and Thompson, 2013).

Empirical Evidence

Include consideration of temporal concordance here

Pre-natal and post-natal Pb exposure affects the hippocampus and the frontal cortex (Schneider et al., 2012). Rats exposed to Pb exhibit microglial activation, and upregulation of the level of IL-1b, TNF-a and iNOS, and these pro-inflammatory factors may cause hippocampal neuronal injury as well as Long Term Potentiation (LTP) deficits, These results suggest a direct link between Pb-induced neuroinflammation, neurodegeneration in hippocampus, and memory deficit (Liu et al., 2012). These effects are reversed by minocycline, an antibiotic which decreases microglial activation, strengthening the link between neuroinflammation, neurodegeneration and memory impairment. In epidemiological studies of adults, cumulative lifetime exposure to Pb has been associated with accelerated declines in cognition (Bakulski et al., 2012). In a study aiming at determining whether serum trace metals are related to abnormal cognition in Alzheimer's disease, it was found that serum Pb levels were significantly negatively correlated with verbal memory scores (Park et al., 2014). Cognitive impaiment was observed in mice exposed to Pb as infants but not as adults, suggesting that a window of vulnerability to Pb neurotoxicity can influence Alzheimer pathogenesis and cognitive decline in old age (Bihaqui et al., 2014). Human Tg-SWDI APP transgenic mice, which over-express amyloid plaques at age of 2-3 months, received oral gavage of 50 mg/kg of Pb once daily for 6 weeks. They showed a significant increase of Abeta in the CSF, brain cortex and hippocampus associated to impaired spatial learning ability, suggesting that Pb facilitates Abeta fibril formation and participate in deposition of amyloid plaques (Gu et al., 2012),

Wozniak et al., (2004) demonstrated that exposure of infant mice to EtOH on a single postnatal day (P7) induced extensive apoptotic neurodegeneration in the developing brain, and subsequent spatial learning and memory impairments that are very severe at P30, less severe if testing is first performed at P75, and minimal in later adulthood. In adulthood, working memory performance was also subtly compromised in EtOH-treated mice in a gender-dependent fashion, with the male EtOH mice being functionally impaired.

Allison et al., (2021) demonstrated that the deleterious effects of AD-related pathophysiology (i.e., higher levels of CSF $ptau_{181}/A\beta_{42}$) on verbal learning and memory performance depend on the degree of global atrophy present. More specifically, individuals with a greater degree of global atrophy evidenced similar rates of decline regardless of the degree of AD pathophysiology present. In contrast, in individuals with larger global brain volumes, the presence of preclinical Alzheimer's disease was associated with steeper declines in verbal learning and memory. These findings suggest that the presence of AD biomarkers, global atrophy, or both global atrophy and AD biomarkers are all associated with greater verbal learning and memory decline in a sample of late middle-aged adults.

Huang et al., (2012) findings indicated that exposure of P7 rats to ketamine leads to accelerated widespread neurodegeneration in the hippocampus. Suppression of p-PKCγ and p-ERK might be involved in neurologic damage, and this neurodegeneration could cause subsequent spatial learning abnormalities in adulthood.

Uncertainties and Inconsistencies

There are some inconsistencies regarding the time of exposure. Some papers clearly show that early Pb exposure increases amyloid and tau pathology and cognitive decline in aging. But few studies have addressed this complex question by using an ad hoc experimental design. Other studies have descibed the effects of lifetime or long-term exposure on cognitive functions but without a precise desciption of exposure onset and duration.

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