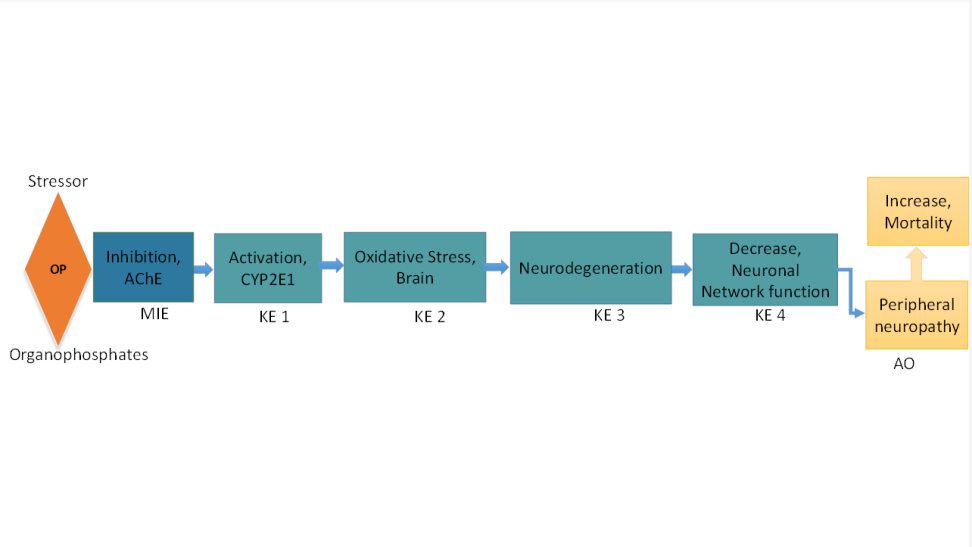
AOP ID and Title:

AOP 450: Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality
Short Title: Organo-Phosphate Chemicals leading to sensory axonal peripheral neuropathy and mortality

Graphical Representation



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Abstract

Organophosphate compounds (OP) such as paraoxon, ethyl parathion, methyl parathion and others are widely used insecticides. OP compounds are also being developed as warfare nerve agents like soman, sarin and tabun etc. The present adverse outcome pathway (AOP) utilizing the existing key events (KEs) and describes the risk associated with a characteristics OP exposure via acetylcholinesterase (AChE) inhibition as molecular initiating event (MIE), which roots a series of KEs that ultimately manifest the adverse outcomes (AO) of sensory axonal peripheral neuropathy and mortality. The MIE of inhibited AChE triggers the KEs: activation of CYP2E1 (aopwiki.org/events/1508), oxidative stress in brain (aopwiki.org/events/1510), neurodegeneration (aopwiki.org/events/352), decreased neuronal network function in adult brain (aopwiki.org/events/618), sensory axonal peripheral neuropathy (aopwiki.org/events/1583) and increased mortality (aopwiki.org/events/351). The OP insecticides inhibits AChE by inhibition of serine esterases and proteases via phosphorylation (Amitai G et al., 1998, Petroianu GA et al., 2001). Farm workers exposed with commonly used OP showed sharp decrease in blood AChE (MIE) level simultaneously over expression of CYP2E1 (KE1) at mRNA level (Sharma RK et al., 2013). CYP2E1-induced oxidative stress (KE2) by over activation of JNK signaling (Schattenberg JM et al., 2014). Literature further supported that oxidative stress causes neurodegeneration (KE3) by targeting different cellular macromolecules including membrane lipid peroxidation, mitochondrial dysfunction and oxidation of protein and nucleic acid (Gandhi S et al., 2012). Thus neurodegeneration and oxidative stress are inherently related phenomena, since microglia and astrocytes are the main sources of reactive oxygen species in CNS degenerative disorders consequently oxidative stress in brain caused neurodegeneration (Mendonca HR et al., 2020, Wakatsuki S et al., 2015, Mishra V et al., 2015, Kim M et al., 2019). Neurodegeneration leads to impairment of retrograde axonal transport that prohibits the growth factor supply to long-range projection neurons, causing synapse loss, and post-synaptic dendrite retraction that leads to decrease of the neuronal network in adult brain (KE 4) (aopwiki.org/relationships/647, Seeley WW et al., 2009). Palop et al (2006) also advocated that neurodegeneration leads to neural network dysfunction. Consequently, loss of neuronal network function in adult brain leads to an important adverse outcome sensory axonal peripheral neuropathy and symptoms include numbness, lack of coordination, impairment of motor nerve, dizziness, burning sensation etc. (Diantonio et al., 2019, Valek L et al., 2019, Persson AK et al., 2016, Topp KS et al., 2000). Thus this AO represents a disparate group of diseases where epidemiological features indicate the potential for a secondary AO of mortality as final adverse outcome (Taverner T et al., 2019, Martyn CN et al., 1997, Hughes RA et al., 1995). Thus this AOP established the connection between OP chemicals and sensory axonal peripheral neuropathy including mortality to endorse predictive toxicology.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	12	Acetylcholinesterase (AchE) Inhibition	AchE Inhibition
2	KE	1391	Activation of Cyp2E1	Activation of Cyp2E1
3	KE	1510	Oxidative Stress in Brain	Oxidative Stress in Brain
4	KE	352	N/A, Neurodegeneration	N/A, Neurodegeneration
5	KE	618	Decreased, Neuronal network function in adult brain	Decreased, Neuronal network function in adult brain
6	AO	1583	Sensory axonal peripheral neuropathy	Sensory axonal peripheral neuropathy
7	AO	351	Increased Mortality	Increased Mortality

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Acetylcholinesterase (AchE) Inhibition	adjacent	Activation of Cyp2E1	Low	Moderate
Activation of Cyp2E1	adjacent	Oxidative Stress in Brain	Moderate	Moderate
Oxidative Stress in Brain	adjacent	N/A, Neurodegeneration	High	High
N/A, Neurodegeneration	adjacent	Decreased, Neuronal network function in adult brain	High	High
Decreased, Neuronal network function in adult brain	adjacent	Sensory axonal peripheral neuropathy	Moderate	Low
Sensory axonal peripheral neuropathy	non-adjacent	Increased Mortality	Low	Low

Stressors

Name	Evidence
Paraoxon	Moderate
Methyl parathion	Moderate
Ethyl Parathion	Moderate
Organophosphates	High

Paraoxon

Paraoxon is an active metabolite of OP pesticide parathion which has been weaponized and used as a chemical-warfare agent that yields high mortality (Laxmikant 2014). Paraoxon is one of the most potent cholinesterase-inhibiting insecticides which is easily absorbed through skin and has been used to study acute and chronic effects of organophosphate intoxication (Deshpande LS et al., 2014). There is strong literature evidence demonstrating inhibition of AChE by paraoxon exposure leading to neurotoxicity. Paroxon inhibition of the enzyme AChE also potentially leads to enhanced glutamate release, diminished GABA uptake, oxidative damage, and neurodegeneration (Farizatto KLG et al., 2017). AChE inhibition and subsequent increased ACh levels can trigger seizures and cause neuronal and excitotoxic damage in the brain. Asymptomatic low-level exposure to such anticholinesterase toxins can also leave the brain vulnerable or even cause it to exhibit neurological problems later in life. Paraoxon exposure initiates a pathogenic cascade involving seizure events and subsequent signs of damage including unique presynaptic vulnerability and associated behavioral deficits (Farizatto KLG et al., 2017). Paraoxon-mediated synaptotoxicity is also associated with enhanced production of oxidative stress as well as integrin adhesion responses (Farizatto KLG et al., 2017).

Organophosphates

Attributes of OPs that produce the MIE and progression of the overall AOP.

Overall Assessment of the AOP

Exposure to OP toxicants is the major source of poisoning worldwide, which affecting approximately 3 million populations early, out of which nearly 15% die as a consequence of poisoning (Farizatto KLG et al., 2017; Eddleston et al., 2008). Exposure route of OP pesticide may be drinking

contaminated water, breathing vapors of the toxicants, or direct exposure to a person's skin with the toxicant. Stressor OPs causes inhibition of MIE (AChE) is well documented in literatures. OP pesticides like parathion and chlorpyrifos exercise their toxicity by irreversible inhibition of AChE (Amitai G et al., 1998). Irreversible inhibition of AChE by OPs in birds, insects, fish, and mammals causes neurotoxicity via characteristics cholinergic toxicity (Cao J et al., 2020; Pundir, C. S et al., 2012). AChE hydrolyses acetylcholine into acetic acid and choline, thus terminates the action of the neurotransmitter acetylcholine. This hydrolysis is inhibited by OP pesticides as OP interfere with the breakdown of acetylcholine by binding to the AChE, which resulted in accumulation of acetylcholine in the nerve synapses and triggered interrupted neurotransmission (Thapa S et al., 2017). Thus OP-induced synaptotoxicity is directly associated with MIE (inhibition of AChE). Inhibition of AChE activates the expression of Cytochrome P450 2E1 i.e CYP2E1 (KE1) as reported in farm workers exposed with OP (Sharma RK et al., 2013). A correlation study between inhibited AChE and CYP2E1 induction in mouse brain is reported by Ma JQ et al., 2016. Enzymology study showed that AChE reactivator's oxime were tested positive for their potential to inhibit CYP2E1 (Spicakova A et al., 2016; Veinlichova A et al., 2009). As previous study reported CYP2E1 a free enzyme with significant pro-oxidant activity is rich source of oxidative stress (Ma JQ, et al., 2016). Thus, in brain the expression of CYP2E1 results in the formation of oxygen radical intermediates and increased oxidative stress (KE2) in the astrocytes of CNS via JNK signaling (Schattenberg JM et al., 2014, Lu Y et al., 2008 Jin, M et al., 2013). Neurodegeneration are characterized by oxidative stress associated disorder with slow and gradual deterioration of structure and function of neurons (Uddin MS et al., 2020). Recent study reported that oxidative stress (KE2) mediated the neurodegeneration (KE3) via mitochondrial dysfunction and excitotoxicity (Uddin MS et al., 2020). Since the main source of oxidative stress in CNS is microglia and astrocytes thus oxidative stresses and neurodegeneration are associated together (Gandhi S et al., 2012). In vitro and in vivo study by Wakatsuki S, et al. (2015) revealed that oxidative stress induced two major pathways of neurodegeneration, neuronal apoptosis and axonal degeneration via phosphorylation dependent ZNFR1 signaling. Previous study suggested that in transgenic mouse model, neurological defects and network dysfunction are associated with neurological diseases (Palop et al., 2006). As per neuropathological inferences, neuroimaging technique, patients suffering from neurodegeneration and the supporting proof from transgenic animal models of neurodegeneration, it's recommended that neurodegeneration is largely associated with decreased neural network function in adult brain. (aopwiki.org/relationships/647, Palop et al., 2007; Seeley WW et al., 2009). Thus neurodegeneration induced loss of neuronal network function in adult brain. Degeneration of injured axons is characteristics features of peripheral neuropathy as animal models demonstrated that blocking axon degeneration can prevent the development of peripheral neuropathy (DiAntonio. 2019). Axonal degeneration of peripheral sensory neurons is a main clinical index of peripheral sensory neuropathy (Liu H et al., 2017). Axonal degeneration induced peripheral neuropathy via self-destructive pathways and mechanistic study in mammalian model highlighted the three distinct pathways i.e wallerian degeneration, apoptosis-induced axon degeneration and pruning-induced axon degeneration (Geden MJ et al., 2016; DiAntonio et al., 2019). As per existing AOP relationship, impaired axonial transport leads to sensory axonal peripheral neuropathy (aopwiki.org/relationships/1835). The loss of neuronal network function in adult brain (KE4) leads to sensory axonal peripheral neuropathy (AO) with symptoms of numbness, lack of coordination, impairment of motor nerve, dizziness, burning sensation etc. (DiAntonio et al., 2019, Valek L et al., 2019, Persson AK et al., 2016, Topp KS et al., 2000). Axonal neuropathy in childhood causes wasting, weakness and delayed development which impacts nervous system and other systemic manifestation, eventually causes mortality (Yiu EM et al., 2012). Peripheral neuropathy is generally irreversible disease with principle of treatment to prevent the progress of disease and related complications (Azhary H et al., 2010, Hicks CW et al., 2019). Since peripheral neuropathy is desperate group of diseases including several common and rare disease thus possess significant contribution in burden of disease and fatal disability in population (Martyn CN and Hughes RA, 1997). As per latest report Peripheral neuropathy was independently allied with mortality in the U.S. population, conclude that decreased sensation in the foot may be an unpredictable risk factor for death and the incident rate of mortality due to peripheral neuropathy is 34.3 % in the general population (Hicks CW et al., 2021). Consequently this evidence reveals the relation between AO (peripheral neuropathy) with enhanced mortality as terminal AO. Thus this AOP can open the route between MIE and AO that is important to scan the human health assessment specially the individual with potential pesticides exposure risk.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Juvenile	High
Adults	Moderate

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

Life Stage Applicability

The key molecular target is the AChE enzyme, which appears to be available in all life stages of different species.

Life Stage	Evidence
Child	High
Adult	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Homo sapiens	Homo sapiens	Moderate	
Rattus norvegicus	Rattus norvegicus	High	
Mus musculus	Mus musculus	High	

Sex Applicability

Sex*	Evidence
Female	Moderate
Male	High

*Study with 44 children for scanning the OP pesticide exposure risk witnessed differences for sex of the child, with male levels higher than female levels (Loewenherz C et al., 1997).

Essentiality of the Key Events

Rationale for essentiality calls:

- MIE: **Inhibition, AChE**: AChE hydrolyses acetylcholine into acetic acid and choline, thus terminates the action of the neurotransmitter acetylcholine. This hydrolysis is inhibited by OP pesticides as OP interfere with the breakdown of acetylcholine by binding to the AChE, which resulted in accumulation of acetylcholine in the nerve synapses and triggered interrupted neurotransmission (Thapa S et al., 2017). Previous studies with vertebrate and invertebrate validate the dependence of AChE activity to the dose of OP and increasing inhibition of AChE in dose dependent manner with OP as reported in fish, birds, nematodes, rodents and mollusk (https://aopwiki.org/events/12). Inhibition of acetylcholine binding at the serine site results in an accumulation of acetylcholine in synapses with muscarinic and nicotinic receptors which resulting in unregulated excitation at junctions (https://aopwiki.org/events/10).
- Key Event 1: **Activation, CYP2E1**: CYP2E1 can define as a P450 monooxygenase that activates the substrates by the addition of oxygen and producing an electrophilic metabolite (https://aopwiki.org/aops/220; Smith, et al. 2016). CYP2E1 in its catalytic cycle produce reactive oxygen species and expressed in the different part of human brain including cortex, cerebellum, hippocampus, thalamus and stratum and produce reactive oxygen species in its catalytic cycle. CYP2E1 is linked with oxidative damage of the brain (Jin, M et al., 2013). Activation of CYP2E1 is ultimately associated with the increase production of ROS (aopwiki.org/relationships/1726). Maneb and Paraquate a neurodegenerative pesticide enhanced the expression of CYP2E1 in male wistar rat which causes oxidative stress (Ahmad I et al., 2014). Activity of CYP2E1 is increasing with drugs, anesthetic agents, pesticides and related disorder (García-Suástegui WA et al., 2017) and linked with AChE inhibition as reported in farm worker (Sharma RK et al., 2013). The highest level of CYP2E1 is found in the mitochondria and the endoplasmic reticulum of rat's brain cells. Expression of CYP2E1 was found in the amygdala and prefrontal cortex in human brain (aopwiki.org/events/1508).
- Key Event 2: **Increased**, **oxidative stress in brain**: The association between activation of CYP2E1 and the formation of ROS, which eventually leads to oxidative stress are well documented (https://aopwiki.org/relationships/1726). Study by Haorah et al. (2008) showed that CYP2E1 indeed produces ROS. Oxidative stress leads to neuronal apoptosis and neuronal degeneration by activating ZNRF1 via epidermal growth factor receptor (EGFR)–mediated phosphorylation (Wakatsuki S et al., 2015). ROS is closely associated to neuronal death in several neurological disorders including Parkinson's and Alzheimer's disease (Guglielmotto M et al., 2009). Acute injury of the brain including brain trauma and cerebral ischemia (Chen SD et al., 2011) or psychiatric disorders like autism, attention deficit, depression and schizophrenia (Michel TM et al., 2012) are also linked with ROS. ROS may target several different substrates and organelle of the cell like DNA, RNA oxidation, or lipid peroxidation (Gandhi S et al., 2012). ROS induced DNA mutation, DNA-protein crosslinks, DNA strand break and transformed purine and pyridine bases is documented (Gandhi S et al., 2012).
- Key Event 3: **Neurodegeneration:** Neurodegeneration is the loss of neuron cells in the brain and spinal cord. The loss of neurons can be define in term of ataxia and dementia i.e. functional loss and sensory dysfunction respectively. Each parts of the brain have their own individual function, thus the neurodegeneration is dependent to individual neuronal loss (Uttara, B et al., 2009)**.** Neurodegeneration is a key aspect of a number of diseases called "neurodegenerative diseases". All of these conditions lead to progressive brain damage and neurodegeneration due to decrease of neuronal function. Neurodegeneration and oxidative stress are inherently related phenomena, since microglia and astrocytes are the main sources of reactive oxygen species in CNS degenerative disorders thus oxidative stress in brain caused neurodegeneration (KE5) (Mendonca HR et al., 2020, Wakatsuki S et al., 2015, Mishra V et al., 2015, Kim M et al., 2019). Hence oxidative stress in brain is the requirement for downstream key event Neurodegeneration.
- Key Event 4: **Decreased, neuronal function in adult brain:** The interaction of neurons is generally consists of a network of several axon terminals connected through synapses to dendrites on other neurons. Once neuron activated, resulted in firing of an electrochemical signal along the axon and it fires only if the total signal received is above firing threshold (aopwiki.org/events/618). In the brain, neurons create a network where the activity of one cell directly influences many others. Neurotransmitters are released at synaptic transmission by presynaptic neuron which further activate the receptors of the postsynaptic neuron (aopwiki.org/events/618). Synaptic transmission relies on: the availability of the neurotransmitter and the binding of the postsynaptic receptor by the neurotransmitter. Once nerve impulse attains at the synapse, the neurotransmitters releases, which influence postsynaptic neuron. The postsynaptic neurons obtain inputs from both excitatory and inhibitory neurons. The excitatory and inhibitory stimuluses all together causing inhibition or "firing" and the inhibition of AChE resulted in decrease of neuronal function (Kolb and Whishaw, 2003). Previous study confirm the inhibition of AChE by OP pesticides paraoxon and chlorpyrifos and study further reported that environmental exposure of chlorpyrifos may leads to sensory peripheral neuropathy in human (Amitai G et al., 1998).
- Adverse Outcome: **Sensory axonal peripheral neuropathy:** The most common neurodegenerative syndrome affecting millions of patients worldwide is Peripheral neuropathy. Peripheral neuropathy affect the motor and autonomic systems are due to axonal loss, consequently loss of neuronal function in adult brain leads to an important adverse outcome Sensory axonal peripheral neuropathy (DiAntonio et al., 2019, Valek L et al., 2019, Persson AK et al., 2016, Topp KS et al., 2000). Depending on the exposure level, some of the acute effects of OPs may leads to excessive secretions, cardiorespiratory depression and life threatening seizures. A number of long-term neurological and psychiatric consequences have been observed in survivors with acute OP exposure (Naughton SX et al., 2018). The symptoms may include sustained attention, motor impairments, psychotic episodes, depressed mood and cognitive flexibility (Steenland K et al., 1994; Pereira EF et al., 2014; Rosenstock et al., 1991; Dassanayake et al., 2007). Reversibility of neuropathy upon discontinuation of treatment is also reported (Brown, T et al., 1991). Peripheral neuropathies mostly affect sensory neurons in a length-dependent manner and therefore characterized by a stocking-and-

glove distribution of the symptoms and numbness and paresthesia like sensory symptoms (Forsyth, P.a., et al. 1997, aopwiki.org/events/1583).

- Adverse Outcome Terminal: **Increased, Mortality** Increased mortality refers to an increase in the number of individuals dying in an experimental replicate group or in a population over a specific period of time (aopwiki.org/events/351). It has been estimated that there are 258,234 (plausible range 233,997 to 325,907) deaths from pesticide self-poisoning worldwide yearly, accounting for 30% (range 27% to 37%) of suicides globally (Gunnell D et al., 2007). Peripheral neuropathies are a disparate group of diseases their contribution to the burden of disease and disability is plausible, however there are limited epidemiological evidence that quantitatively connecting the previous AO to mortality rates with regard to the final adverse outcome (Taverner T et al., 2019, Martyn CN et al., 1997, Hughes RA et al., 1995). As per latest report Peripheral neuropathy was independently allied with mortality in the U.S. population, concluded that decreased sensation in the foot may be an unpredictable risk factor for death thus the incident rate of mortality due to peripheral neuropathy is 34.3 % in the general population (Hicks CW et al., 2021). Study in vertebrate and invertebrate showed that 70 to 80 % inhibition of AChE in brain leads to significant increase in mortality via accumulation of ACh (Russom CL et al., 2014).

Weight of Evidence Summary

"'Biological Plausibility"'

OP induced inhibition of AChE leads to accumulation of acetylcholine (ACh) at the cholinergic synaptic clefts, resulting in to long-term activation of the nicotinic and muscarinic ACh receptors (AChR) and overstimulation of cholinergic neurons with hyper excitation and seizures (Faria, M et al., 2015; Peña Llopis, S. & Pena, 2005). OP induced seizures can grow to status epilepticus causing severe damage of brain (Weissman, B. A. & Raveh, L et al., 2008). Inhibition of AChE is associated with wide range of adverse effect including fatality, hence it's dependent to concentration and half-life of OP dose. Major adverse effects with accumulation of ACh in peripheral system includes glandular secretions, skeletal muscle twitching, smooth muscle contractions and flaccid paralysis, simultaneously centrally mediated impacts associated with learning, memory and behavioral impairments (https://www.epa.gov/sites/production/files/2015-07/documents/cholin.pdf).
Oxidative stress is the key mechanisms involved in OP induced neurotoxicity, which can caused by over-production of reactive oxygen species (ROS) in the electron transport system and metabolism of agents by cytochrome P450 (Farkhondeh T et al., 2020). Thus the oxidative stress is inducer of neuronal apoptosis and axonal degeneration. Since it activate the ZNRF1 by inducing epidermal growth factor receptor (EGFR)–mediated phosphorylation at the 103rd tyrosine residue thus the up-regulation of ZNRF1 activity by oxidative stress leads to neuronal apoptosis and Wallerian degeneration (Wakatsuki S et al., 2015). As per neuropathological outcomes and neuroimaging of patients suffering from neurodegeneration as well as complementary evidence from transgenic animal models of neurodegeneration, it has been recommended that neurodegeneration is related to neural network dysfunction (aopwiki.org/relationships/368; Palop et al., 2007b; Seeley WW et al., 2009). Neurodegeneration leads to impairment of retrograde axonal transport that prohibits the growth factor supply to long-range projection neurons, causing synapse loss and post-synaptic dendrite retraction that leads to decreases of the neuronal network (Seeley WW et al., 2009) (https://aopwiki.org/relationships/647). Defective axonal transport are the major cause for peripheral neuropathies as the length-dependent and distal neurologic deficits observed in patients with axonal loss, which are often linked to axonal transport defects (https://aopwiki.org/relationships/1835 ; Rowinsky, E.K et al., 1995). Mutations linked to axonal transport are known cause of Charcot-Marie-Tooth disease (a peripheral neuropathy) (Zhao, C et al., 2001). Thus the function of axon survival and axon degeneration proteins play an important role in the mechanistic insight. Usually, the clinical presentation reflects an axonal peripheral neuropathy with glove-and-stocking distribution sensory loss, collective with the features indicative of nerve hyper excitability includes paresthesia, dysesthesia, and pain. Symptoms like proprioceptive and motor effects may be prolonged, severe disabling and adversely affect activities of daily living and quality of life (Bhutani M et al., 2010).

"'Concordance of dose-response relationships:"'

Impairments in axonal transport could be detected even at concentrations that did not inhibit AChE activity and they were not blocked by cholinergic receptor antagonists (Naughton SX et al., 2018). Both neuronal culture and animal studies have reported that OP- induced axonal transport deficits as a potential non-cholinesterase mechanism for the long-term deleterious neurological effects (Naughton SX et al., 2021). Physostigmine was establish to strongly inhibit the purified eel AChE with an IC_{50} of 0.02 µM but not impaired axonal transport (Naughton SX et al., 2021).

"'Temporal concordance among the key events and adverse effect:"'

Sufficient literature evident that around 25 -32% of the US veterans deployed in Persian Gulf War was reported with chronic health symptoms like headache, respiratory problems, musculoskeletal pain and a number of neurological and neuropsychiatric illness including cognitive defects (Naughton SX et al., 2018; Sullivan et al., 2018), inline with exposure of OP based inhibitors of AChE (Winkenwerder, 2003). Depending to exposure level, the effect of OP exposure may include cardiorespiratory depression, life threatening seizures and long term neurological and psychiatric consequences in survival including motor impairment (Naughton SX et al., 2018; Pereira et al., 2014). As per previous report the animal models have been largely appreciated to reveal the effects of anti- AChE abuses and their connection to brain damage in humans. Harrison et al., (2004) establish that OP (Paraoxon) caused seizures in guinea pigs similar to those caused by the nerve gas soman in humans. Similarly, the anti-AChE strength of Paraoxon was similar in macaques and humans (Worek et al., 2011). Moreover, Rosenberg et al., (2017) showed that anti- AChE exposed macaques presented severe signs of toxicity such as fasciculations, miosis, salivation and convulsions including fatality in less than seven hours of application.

"'Consistency:"'

The cholinergic phase typically last within 48 hrs including medical emergency in intensive care unit (Senanayake N et al., 1987). OP induced inhibition of AChE caused accumulation of ACh at muscarinic sites and resulted in secretions, bronchoconstriction, blur vision and bradycardia. Accumulation of ACh at nicotinic sites triggered flaccid paralysis, insomnia, confusion, drowsiness convulsions, coma, and respiratory depression (PMCID: PMC6357250, *EJIFCC*. 1999). Peripheral neuropathy is the most common neurodegenerative disorder, caused by damaging to peripheral nerves and is an important cause of neuropathic pain including hyperalgesia, allodynia, and dysesthesias (DiAntonio et AL et al., 2019). Acute exposure of organophosphate induced delayed neuropathy is well reported which is characterized by distal degeneration of axons of CNS and PNS. Non excitability of the nerve with electromyographical signs of denervation has been noticed with progression of disease and the experimental date with human shows recovery in the young (Lotti M., 2005). Transcutaneous intoxication with OP showed the nicotinic effect in young children (Pavlovic M., 2015). A study by Yonggang Li et al (2012) reported that Adult male Sprague Dawley rats treated with acute exposure of OP (diisopropylfluorophosphate) showed that almost all animals developed severe seizures and died within 30 minutes but with delayed neuronal injury model, animal survived and delayed neuronal injury was observed till 4-72 hrs (Li Y et al., 2011). Furthermore the role of oxidative stress in neuronal injury and oxidative stress-mediated protein damage in the brains of diisopropylfluorophosphate -intoxicated rats is also documented (Li Y et al., 2012).

"'Uncertainties, inconsistencies, and data gaps:"'

Cholinergic hyper stimulation which affect nervous system via AChE inhibition is very common mode of action of OP (Pope CN et al., 1999), simultaneously the mouse lacking AChE also showed hyper sensitive of OP via non AChE mechanisms of action (Duysen EG et al., 2001). Unfortunately, the very limited evidence are available for the measurement of AChE inhibition in peripheral neural tissues or neuro-effector junctions. Reductions in neural AChE activity may not always associated with obvious clinical signs because critical functions of those specific neurons may not be sufficiently evaluated to identify associated changes or tolerance developed. The time at which potential functional effects are evaluated may also contribute to an apparent lack of concordance between functional effects and cholinesterase inhibition. Thus it is difficult to determine, with accuracy or consistently, the degree of cholinesterase inhibition that will cause specific physiological or behavioral changes (https://www.epa.gov/sites/production/files/2015-07/documents/cholin.pdf). The existing evidence mostly focused on the whole brain AChE activity, but not usually regional brain measurements, or time-course data, mainly with acute exposures. Thus this is a major limitation, and also the distribution of cholinergic pathways, their concentration and molecular form of AChE in different brain regions is not uniform. Thus, whole brain measurements of AChE inhibition may tell little or no change in activity, while masking significant changes in specific brain regions linked with particular cholinergically-mediated functions can tell more (https://www.epa.gov/sites/production/files/2015-07/documents/cholin.pdf).

Quantitative Consideration

OP is commonly used as insecticides because it inhibits the AChE of insects (Tomlin C et al., 1994) and the same characteristic mode of cholinergic toxicity has been reported in human (Taylor P et al., 1996). Previous study showed that OP is more effective inhibitor of AChE than neuropathy target esterage (NTE) which causing delayed polyneuropathy at higher dose (Lotti M et al., 1992; Moretto A et al., 1998), simultaneously patients with low level repeated exposure of OP developed sensory neuropathy (Kaplan JG et al., 1993; Moretto A et al., 1998). Inhibition of AChE (MIE) is considered a vital event in the mechanism of cholinergic toxicity, the AChE-inhibiting OP causes a broad range of adverse effects including sensory, cognitive, and psychological effects (Reigart, R. and Roberts, 1999). Decreasing AChE level and increasing CYP2E1 (KE1) is reported in farm worker (Sharma RK et al., 2013). CYP2E1-induced oxidative stress (KE2) (Schattenberg JM et al., 2014) causes neurodegeneration (KE3) (Gandhi S et al., 2012) that leads to dysfunction of neuronal function (Palop et al., 2006). Subsequently, the loss of neuronal network function (KE4) leads to an adverse outcome of sensory axonal peripheral neuropathy (AO) and the symptoms may include lack of coordination, impairment of motor nerve, dizziness, burning sensation, numbness etc. (DiAntonio et al., 2019). This desperate group of diseases due to peripheral neuropathy causes fatal disability and mortality (AO) in population (Martyn CN and Hughes RA, 1997). As per latest report Peripheral neuropathy was self-sufficiently allied with mortality in the U.S. population, concluded that decreased sensation in the foot may be an unpredictable risk factor for death and the rate of mortality associated with peripheral neuropathy is 34.3 % in the general population (Hicks CW et al., 2021). Thus clinical symptoms with behavioral or physiological effects is the most direct evidence of the potential adverse consequences of human exposure to anticholinesterase pesticides like OP (https://www.epa.gov/sites/production/files/2015-07/documents/cholin.pdf).

Considerations for Potential Applications of the AOP (optional)

The potential application of this AOP includes risk assessment in predictive modeling of OP pesticide toxicity via AChE dependent pathways. Thus this AOP is an attempt to create the mechanism of organophosphorus (OP) pesticides induced sensory peripheral neuropathy and mortality as an adverse outcome. Supplementary evidence may help this AOP to fully elucidate the mechanistic approach and the neurotoxic potential of OP pesticide.

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Appendix 1

List of MIEs in this AOP

Event: 12: Acetylcholinesterase (AchE) Inhibition

Short Name: AchE Inhibition

Key Event Component

Process	Object	Action
acetylcholinesterase activity	acetylcholinesterase	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	MolecularInitiatingEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	MolecularInitiatingEvent
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	MolecularInitiatingEvent
Aop:405 - Organo-Phosphate Chemicals induced inhibition of AChE leading to impaired cognitive function	MolecularInitiatingEvent
Aop:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	MolecularInitiatingEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Stressors

Name
Organophosphates
N-methyl Carbamates

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term
nervous system

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

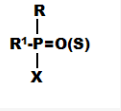
- Organophosphate and carbamate insecticides are prototypical AChE inhibitors. The OP and carbamate pesticides were synthesized specifically to act as inhibitors of AChE, with OPs developed from early nerve agents (e.g., sarin) and carbamate pesticides based on the natural plant alkaloid physostigmine (Ecobichon 2001).
- A positive and significant correlation between the log of the Eserine IC50 (in vitro) for AChE inhibition and the log Km value for the AChE in the fish and crustacea species has been reported, explaining 92% of the variation in enzyme inhibition (Monserrat and Bianchini, 2001). Similar success was found in relating the rate constants for inhibition of AChE in housefly and the pseudo first-order hydrolysis rate constant for active forms of OPs (Fukuto 1990).

- The open literature includes many studies on vertebrate and invertebrate species that demonstrate a clear dependence of AChE activity on the dose or concentration of the substance with increased concentrations leading to an increase in the inhibition of AChE (e.g., fish (Karen et al., 2001), birds (Hudson et al., 1984 (see dimethoate and disulfoton), Grue and Shipley 1984; and Al-Zubaidy et al., 2011); cladocera (Barata et al., 2004); nematodes (Rajini et al., 2008); rodents (Roberts et al., 1988; and mollusk (Bianco et al., 2011)).
- The open literature includes many studies on vertebrate and invertebrate species that demonstrate a clear relationship between increasing AChE inhibition as duration of exposure increases (e.g., amphibians (Venturino et al., 2001); fish (Rao 2008; Ferrari et al., 2004); insects (Rose and Sparks 1984); birds (Ludke 1985; Grue and Shipley 1984); annelids (Reddy and Rao 2008); cladocera (Barata et al., 2004)).
- Rao et al. 2008 exposed the estuarine fish Oreochromis mossambicus to a 24 h LC50 concentration of chlorpyrifos and reported that it took 6 hr to reach >40% AChE inhibition and 24 hr to reach 90% AChE inhibition. It took >100 days to recover to normal AChE levels when fish were placed in clean water.
- A time course study of earthworms (Eisenis foetida) exposed to the 48 hr LC50 of profenofos found a significant relationship (between increases in percent inhibition of AChE and increase in time of exposure from 8-48 hrs (Chakra Reddy and Rao 2008).

Organophosphates

The MIE, AChE inhibition, is triggered via electrostatic interaction at the anionic site of the enzyme and binding with the serine hydroxyl group at the esteratic site of AChE (Wilson 2010; Fukuto 1990). Organophosphate pesticides attach to the AChE via an 'irreversible' phosphorylation of the enzyme. Note that the use of the term 'irreversible' relates to the relative rate at which the phosphorylation occurs since acetylcholine and organophosphates both form covalent bonds with the enzyme. The phosphorylated form may persist for up to a week if it has undergone an 'aging' process; i.e., the organophosphate has undergone a dealkylation, thereby strengthening the bond between the OP and the enzyme (Mileson et al. 1998; Kropp and Richardson 2003; Sogob and Vilanova 2002). Certain steric and electronic requirements must be met in order for an organophosphate to inhibit AChE. For instance, organophosphates require a leaving group sufficiently electronegative to ensure the formation of a reactive electrophile (Fukuto 1990; Sogob and Vilanova 2002; Schűűrmann 1992). Substances with subtle structural differences can result in major changes in AChE inhibition capabilities. For example, OPs having identical R and R1 alkyl groups display decreasing AChE inhibition as the R / R1 carbon chain increases from a single carbon to a propyl moiety, with the latter resulting in an ineffective AChE inhibitor (Fukuto 1999).

Metabolism also plays an important role in the potency of organophosphates. For instance, organophosphates in the phosphorothionate and phosphorodithioate families (i.e., P=S) must undergo metabolic activation, via cytochrome P450-based monoxygenases, to an oxon form in order to inhibit AChE effectively (Fukuto 1990).

Base Structure (OP)	Configuration
	R: A simple alkyl (e.g., methyl or ethyl group) or aryl group bonded to either an oxygen or sulfur that is directly bonded to the phosphorous; R1: Methoxy, ethoxy, ethyl, phenyl, amino, substituted amino, or alkylthio group; X: Leaving group that is or contains an electronegative moiety (e.g., phenoxy or aromatic group containing hetero atoms, substituted thioalkyl, or substituted alkoxy groups); O: Oxons are direct acting S: Thiophosphates require metabolic activation to the oxon form in order to be active AChE inhibitors

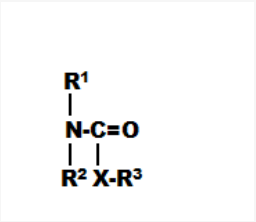
Evidence exists that immature life stages in mammals and birds may be more sensitive to organophosphate pesticides (see Grue et al., 1997; Grue et al., 1983; Grue et.al; 1981). It has been suggested that this may be related to the amount of pesticide ingested in relation to body size (Ludke et al, 1975), but there is direct data in rats showing that differential sensitivity to OPs is determined at least in part by inadequate detoxification in the young (Moser, 2011). OP detoxification is highly dependent on enzymes such as A-esterases (paraoxonases, PON) and carboxylesterases (e.g., Benke and Murphy, 1974; Furlong, 2007; Sterri et al., 1985; Vilanova and Sogorb, 1999), which are present at lower levels in the young (e.g., Chanda et al., 2002; Mendoza, 1976; Mortensen et al., 1996; Moser et al., 1998).

N-methyl Carbamates

Carbamates trigger AChE inhibition through electrostatic interactions at the enzyme's anionic site and binding with the serine hydroxyl group at the esteratic site (Wilson 2010; Fukuto 1990). Carbamates, which were originally based on the plant alkaloid physostigmine, attach to the AChE via a 'reversible' carbamylation. Note that the use of the term 'reversible' relates to the relative rate at which the carbamylation occurs since acetylcholine and carbamates both form covalent bonds with the enzyme. Certain steric and electronic requirements, as well as the leaving group on the pesticide, are critical to the likelihood that the methyl-carbamate will inhibit AChE (See Figure).

Metabolism also plays a role in the potency of some carbamates. Select procarbamates require metabolism to form an active AChE inhibitor (e.g., carbosulfan must be metabolized to carbofuran), or are made more potent via metabolism (e.g., aldicarb oxidation to the more toxic sulfoxide form) (Sogob and Vilanova 2002; Stenersen 2004).

Base Structure (Carbamate) Configuration



R1: Methyl group

R2: Hydrogen group;

XR3: Leaving group that is an aryloxy or oxime;

pKa: For oxime and substituted phenols, a pKa in the range of 10 ensures carbamylation;

Carbamates must 'fit' in the enzyme active site to be effective inhibitors

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

AChE is present in all life stages of both vertebrate and invertebrate species (Lu et al 2012).

- Acetylcholinesterase associated with cholinergic responses in most insects is coded by the ace1 gene and in vertebrates by the ace gene (Lu et al 2012; Taylor 2011.
- Plants have AChE but it is most likely involved in regulation of membrane permeability and the ability of a leaf to unroll (Tretyn and Kendrick 1991).
- The primary amino acid sequence of the AChE enzyme is relatively well conserved across vertebrate and invertebrate species, suggesting that chemicals are likely to interact with the enzyme in a similar manner across a wide range of animals. From the sequence similarity analyses, the taxonomic domain of applicability of this MIE likely includes species belonging to many lineages, including branchiopoda (crustaceans, e.g., daphnids), insecta (insects), arachnida (arachnids, e.g., spiders, ticks, scorpions), cephalopoda (molluscans, e.g., octopods, squids), lepidosauria (reptiles, e.g., snakes, lizards), chondrichthyes (cartilaginous fishes, e.g., sharks), amphibia (amphibians), mammalian (mammals), aves (birds), actinopterygii (bony fish), ascidiacea (sac-like marine invertebrates), trematoda (platyhelminthes, e.g., flatworms), and gastropoda (gastropods, e.g., snails and slugs) Species within these taxonomic lineages and others are predicted to be intrinsically susceptible to chemicals that target functional orthologs of the daphnid AChE (Russom, 2014).
- Advanced computational approaches such as crystal structures of the enzyme and transcriptomics have provided empirical evidence of the enzyme structure, relevant binding sites, and function across species (Lushington et al., 2006; Lu et al., 2012; Wallace 1992).

Studies have found that AChE activity increases as the organism develops.

- Prakesh and Kaur 1982 looked at AChE inhibition across three insect species; controls and those exposed to DDVP. They saw little difference in the larval stages but did see increased inhibition in pupal and adult stages (greatest inhibition).
- Karanth and Pope 2003 looked at AChE and acetylcholine synthesis in rat striatum in controls and animals exposed to 0.3 and 1 times the maximum tolerated dose. Although these doses are below the lethal concentrations and they mention that not observed cholinergic responses were observed, they do provide differences related to life stages of the rodents.
- Grue et al 1981 present baseline (no toxicity exposure) in wild starlings (both sexes) of brain cholinesterase and found activity increased as birds aged from 1-20 days until it reached a steady state at adulthood.
- A study with Red Flour Beetle found that the gene associated with cholinergic functions (Ace1) was expressed at all life-stages, with increases as the organism developed from egg to larva to pupa to adult. (Lu et al., 2012 cited in Russom et al 2014.)
- In mammals and birds, studies have determined that skeletal muscles of immature birds and mammals contain both butyrylcholinesterase and AChE, with butyrylcholinesterase decreasing and AChE increasing as the animal develops (Tsim et al. 1988; Berman et al, 1987).
- Another study found that changes in AChE within the developing pig brain were dependent on the area of the brain, and life stage of the animal, with significant decreases in activity within the pons and hippocampus from birth to 36 months, and no significant change in activity in the cerebellum, where activity increased up to four months of age, leveling off thereafter (Adejumo and Egbunike, 2004).

Key Event Description

"Acetylcholinesterase is found primarily in blood, brain, and muscle, and regulates the level of the neurotransmitter ACh [acetylcholine] at cholinergic synapses of muscarinic and nicotinic receptors. Acetylcholinesterase features an anionic site (glutamate residue), and an esteratic site (serine hydroxyl group) (Wilson, 2010; Soreq, 2001). In response to a stimulus, ACh is released into the synaptic cleft and binds to the receptor protein, resulting in changes to the flow of ions across the cell, thereby signaling nerve and muscle activity. The signal is stopped when the amine of ACh binds at the anionic site of AChE, and aligns the ester of ACh to the serine hydroxyl group of the enzyme. Acetylcholine is subsequently hydrolyzed, resulting in a covalent bond with the serine hydroxyl group and the subsequent release of choline, followed by a rapid hydrolysis of the enzyme to form free AChE and acetic acid (Wilson, 2010; Soreq, 2001)." [From Russom et al. 2014. Environ. Toxicol. Chem. 33: 2157-2169]

Molecular target gene symbol: ACHE

KEGG enzyme: EC 3.1.1.7

How it is Measured or Detected

- Direct measures of AChE activity levels can be made using the modified Ellman method, although selective inhibitors that remove other cholinesterases not directly related to cholinergic responses (e.g., butyrylcholinesterase) are required [45,46].
- Radiometric methods have been identified as better for measuring inhibition because of carbamylation (carbamate exposure) [20,46,47].
- TOXCAST: NVS_ENZ_hAChE
- A direct measure of cholinesterase activity levels can be made within the relevant tissues after in vivo exposure, specifically the brain as well as red blood cells in mammals. Some analytical methods used to measure cholinesterase activity may not distinguish between butyrylcholinesterase, which is found with AChE in plasma and some skeletal and muscle tissues. Although the structure of butyrylcholinesterase is very similar to AChE, its biological function is not clear, and its activity is not associated with cholinergic response covered under this AOP (Lushington et al., 2006). Therefore experimental procedures used to measure cholinesterase as well as the tissue analyzed should be considered when evaluating studies reporting AChE inhibition (Wilson 2010; Wilson and Henderson 2007). For measuring AChE levels, the Ellman method is recommended with some modifications (Ellman et al., 1961; Wilson et al., 1996) while radiometric methods have been identified as better for measuring inhibition due to carbamylation (carbamate exposure) (see Wilson 2010; Wilson et al., 1996; Johnson and Russell 1975).

- In order to effectively bind to the AChE enzyme, thion forms of OPs (i.e., RO)3P=S) must first undergo a metabolic activation via mixed function oxidases to yield the active, oxon form (Fukuto 1990). Estimating the potential toxicity in whole organisms based on in vitro data may be problematic since metabolic activation may be required (e.g., phosphorothionates) and may not be reflected in the in vitro test result (Guo et al. 2006; Lushington et al. 2006).
- Typically, carbamates do not require metabolic activation in order to bind to the enzyme, although some procarbamates (e.g., carbosulfan) have been developed that are not direct inhibitors of AChE, but take advantage of metabolic distinctions between taxa, resulting in a toxic form in invertebrates (e.g., carbofuran) but not vertebrate species (Stenersen 2004). Therefore in vitro assays measuring AChE inhibition for procarbamates in invertebrate species will not account for metabolic activation and therefore may not represent the actual enzyme activity.

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List of Key Events in the AOP

Event: 1391: Activation of Cyp2E1

Short Name: Activation of Cyp2E1

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:220 - Cyp2E1 Activation Leading to Liver Cancer	MolecularInitiatingEvent
Aop:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	KeyEvent

Stressors

Name
Chloroform
Acetaminophen
furan
Ethanol
acetone

Biological Context

Level of Biological Organization

Molecular
Level of Biological Organization

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

A variety of substrates have been described (Lieber 1997, Tanaka, et al. 2000). There are >85 known Cyp2E1 substrates. They are low molecular weight compounds, including: molecular oxygen, acetone (Bondoc, et al. 1999), acetaminophen (Lee, et al. 1996, Zaher, et al. 1998), carbon tetrachloride (Wong, et al. 1998), pyrazole, vinyl chloride, furan, chloroform, ethanol (Bardag-Gorce, et al. 2000), benzene (Powley and Carlson 2001), acrylonitrile (El Hadri, et al. 2005), trichloroethylene (Kim and Ghanayem 2006), aniline, N-nitrosodimethylamine, N-nitrosodiethylamine, diethylnitrosamine, thioacetamide (Chilakapati, et al. 2007), urethane (Hoffler, et al. 2003, Hoffler and Ghanayem 2005), and toluene.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rodents	rodents	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Taxonomic applicability: The Cyp2E1 gene is present across a variety of taxa including humans and primates, mice and rats. AceView (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html) indicates high levels of Cyp2E1 expression from RNA-seq experiments in liver across primate species. Cype2E1 is also present in frogs (Fort, et al. 2003, Saito, et al. 1997) and fish (Howarth, et al. 2011).

Life stages: Studies are primarily on adult liver tissues.

Sex applicability: Cyp2E1 is expressed in both males and females.

Key Event Description

Cyp2E1 is a membrane-bound monooxygenase that is primarily located in zone 3 hepatocytes (Ingelman-Sundberg, et al. 1988, Tsutsumi, et al. 1989). Although it is also expressed in other tissues (http://www.genecards.org/cgi-bin/carddisp.pl?gene=CYP2E1), the body of literature on CYP2E1 is focussed on measurement in liver. CYP2E1 is primarily located in the endoplasmic reticulum, but can also be present in the mitochondria. It is a phase I metabolism enzyme that catalyzes the oxidation of low molecular weight substrates. Unlike most cytochrome P450 enzymes, Cyp2E1 is constitutively expressed (i.e., its expression is not transcriptionally controlled by substrate-bound nuclear receptors). Alternatively, exposure to a substrate increases its activity through post-translational stabilization of the molecule. Thus, the presence of substrate significantly increases the half-life of the Cyp2E1 enzyme thereby allowing it to be active for a longer period of time (Gonzalez 2007, Song, et al. 1989). The sustained activation of Cyp2E1 due to the presence of the chemical substrate is required for this MIE to produce downstream adverse effects.

Cyp2E1 is also regulated by the ubiquitin-proteasome pathway and the involvement of hsp-based chaperone (Morishima et al. 2005); however, this mechanism of regulation is not discussed further herein.

How it is Measured or Detected

- **Mixed function oxidase catalytic activity.** These assays have been thoroughly reviewed by Cederbaum (2014). The paper describes preparation of microsomes from both liver homogenates and cell cultures for testing Cyp2E1 activity. Briefly, the ratio of 6-hydroxychlorzoxazone/chlorzoxazone can be used to estimate levels of CYP2E1 in humans (Girre, et al. 1994). In addition, the oxidation of para-nitrophenol (PNP) to para nitrocatechol is an efficient and relatively specific assay to determine catalytic activity dependent on CYP2E1 [e.g., (Koop 1986, Koop, et al. 1989, Reinke and Moyer 1985)]. Other assays are described within the review article by Cederbaum.
- **Western blot or Immunohistochemistry.** Following chemical treatment, Cyp2E1 protein levels should increase if it is involved in the metabolism of that substrate. Western blot (of protein extracted from liver or cultured cells) or immunohistochemistry (of fixed liver or cultured cells) using anti-Cyp2E1 antibodies is the most straightforward approach for directly measuring increased levels of Cyp2E1.
- **HepG2 cells.** A compound's Cyp2E1-dependence can be determined by comparing toxic effects in HepG2 versus HepG2-E47 cells. HepG2 cells are immortalized human hepatoma cells that do not express Cyp2E1; whereas, HepG2-E47 cells over-express Cyp2E1 (by recombinant retroviral infection). Chemicals that are metabolically activated by Cyp2E1 will cause cytotoxicity and oxidative stress in the E47 cells only. Toxicity can be blocked by treatment with antioxidants or Cyp2E1 inhibitors. Toxicity is exacerbated when glutathione is depleted (Wu and Cederbaum 2005) (e.g., ethanol (Cederbaum, et al. 2001, Chen and

Cederbaum 1998, Chen, et al. 1998, Dai, et al. 1993).

- Measurement of chemical oxidation by Cyp2E1 in liver microsomes; described in the methodology review by Cederbaum (Cederbaum 2014). Reactions use specific probes to confirm that the compound undergoes oxidation, and that this oxidation reaction is catalyzed by Cyp2E1. See also: (Koop 1986, Koop, et al. 1989, Reinke and Moyer 1985).
- **Cyp2E1 knock-out mouse**. Chemical exposures in knockout mice are conducted and the production of the anticipated metabolites is measured. Lack of metabolite production indicates that Cyp2E1 is required for the chemical's metabolism. Effects in knock-out mice are always measured in reference to wild-type (control) mice, which allows investigators to attribute the altered phenotype to the gene that has been knocked-out. Studies in Cyp2E1 knockout mice indicate the following chemicals interact with it: carbon tetrachloride (Wong, et al. 1998), acetone (Bondoc, et al. 1999), benzene (Powley and Carlson 2001), thioacetamide (Chilakapati, et al. 2007), trichloroethylene (Kim and Ghanayem 2006), acrylonitrile (El Hadri, et al. 2005), urethane (Hoffler, et al. 2003, Hoffler and Ghanayem 2005), acetaminophen (Lee, et al. 1996, Zaher, et al. 1998), and ethanol (Bardag-Gorce, et al. 2000).
- **Humanized Cyp2E1 mice**. Two transgenic mice with human Cyp2E1 have been created. The first mouse reproduces and develops normally, and demonstrates Cyp2E1-dependent toxicity (Morgan, et al. 2002). However, these mice express human and endogenous Cyp2E1, which is not ideal. A true 'humanized' Cyp2E1 transgenic mouse was produced by the Gonzalez lab in which the endogenous Cyp2E1 gene was replaced with the human Cyp2E1 gene (Cheung, et al. 2005, Cheung and Gonzalez 2008). Studies in these mice are conducted in order to provide evidence that the Cyp2E1-dependent effects observed in experimental animals will also occur in humans.
- **2-Piperidone.** 2-Piperidone is a newly proposed biomarker of Cyp2E1 activity that is detected in urine (Cheng, et al. 2013).

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Event: 1510: Oxidative Stress in Brain

Short Name: Oxidative Stress in Brain

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:260 - CYP2E1 activation and formation of protein adducts leading to neurodegeneration	KeyEvent
Aop:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	KeyEvent
Aop:501 - Excessive iron accumulation leading to neurological disorders	KeyEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Key Event Description

Oxidative stress is the imbalance between ROS and defence mechanisms against these ROS. Due to this imbalance the concentration of ROS can rise in the cells. The oxidizing free radicals can cause damage in the cell, at the DNA but also on multiple proteins. Nrf2 is an example of an defence mechanism against ROS.

How it is Measured or Detected

One way to show oxidative stress in cells is to measure the ROS level. ROS generation can be determined in neuron cells. DHE, a small-molecule ROS probe, can be used for visualizing ROS generation with the use of an epifluorescence microscope. DHE is a direct way of measuring ROS, an indirect way is the measurement of glutathione depletion. The ratio between glutathione and oxidized glutathione can be determined, which shows indirectly whether the ROS level is increased. When oxidized glutathione is present in a higher concentration the ROS level is increased. Finally the

expression level of Nrf2 can be determined in cells, with the use of a western blot analysis and antibodies for Nrf2. When the expression level of Nrf2 is much higher than in a control cell it indirectly shows that there is an increase in the concentration of ROS.

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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
Organ term

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
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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Event: 618: Decreased, Neuronal network function in adult brain

Short Name: Decreased, Neuronal network function in adult brain

Key Event Component

Process	Object	Action
synaptic signaling		decreased

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:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	KeyEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

brain

Domain of Applicability

The ability to process complex spatiotemporal information through neuronal networking is a fundamental process underlying the behaviour of all higher organisms. The most studied are the neuronal networks of rodents (e.g Reig et al., 2015) and primates (e.g. Wang and Arnsten, 2015) and extremely large amount of the published data exist to support this topic. Invertebrates hold neural circuitries in various degrees of complexity and there are studies describing how neurons are organized into functional networks to generate behaviour. (Wong and Wong, 2004; Marder, 1994).

Key Event Description

Biological state:

In the brain, neurons never work alone. They create a network where the activity of one cell directly influences many others. Each neuron is a specialized cell and when activated, it fires an electrochemical signal along the axon. A neuron fires only if the total signal received at the cell body from the dendrites exceeds a certain level (the firing threshold). The strength of the signal received by a neuron (and therefore its chances of firing) critically depends on the efficacy of the synapses. Each synapse actually contains a synaptic cleft with neurotransmitter that transmits a signal across the gap. During synaptic transmission neurotransmitters are released by a presynaptic neuron and bind to and activate the receptors of the postsynaptic neuron in response to a threshold of action potential. Synaptic transmission relies on: the availability of the neurotransmitter; the release of the neurotransmitter by exocytosis; the binding of the postsynaptic receptor by the neurotransmitter; the functional response of the postsynaptic cell; and the subsequent removal or deactivation of the neurotransmitter. Neurons form complex networks of synapses through which action potentials travel. When the nerve impulse arrives at the synapse, it may cause the release of neurotransmitters, which influence another (postsynaptic) neuron. The postsynaptic neurons receive inputs from many additional neurons, both excitatory and inhibitory. The excitatory and inhibitory influences are summed (neural summation) resulting in inhibition or "firing" (i.e., generate an action potential) if the threshold potential has been reached. The voltage at which an action potential is triggered happens if enough voltage-dependent sodium channels are activated and the net inward sodium current exceeds all outward currents (Kolb and Whishaw, 2003). Therefore, at the beginning of the action potential, the Na+ channels open and Na+ moves into the axon, causing depolarization. Re-polarization occurs when the K+ channels open and K+ moves out of the axon. This creates a change in polarity between the outside of the cell and the inside. The impulse travels down from the axon hillock in one direction only, to the axon terminal. Here, the neurotransmitter is released releasing neurotransmitter at the synaptic cleft to pass along information to another adjacent neuron. Excitatory inputs bring a neuron closer to a firing threshold, while inhibitory inputs bring the neuron farther from threshold. An action potential is an "all-or-none" event; neurons whose membranes have not reached threshold will not fire, while those that do, will fire. One of the most influential researchers into neurological systems (Donald Hebb) postulated that learning consisted principally in altering the "strength" of synaptic networking. Recent research in cognitive science, in particular in the area of non-conscious information processing, have further demonstrated the enormous capacity of the human mind to learn simple input-output co-variations from extremely complex stimuli. Consequently, the neurodegeneration and cell death disrupt the natural rhythms of brain network communication. Cognitive disorders are primarily associated with dysfunction of the neurons of the prefrontal cortex, hippocampus and with changes mainly in NMDARs function (Wang et al, 2015).

Biological compartments:

The interface through which neurons interact with their neighbours usually consists of several axon terminals connected via synapses to dendrites on other neurons. If the hippocampal or cortical neurons are damaged or killed by the over-activation of receptors for the excitatory neurotransmitter glutamate, such as the NMDA, kainate and AMPA receptors, the neuronal networking and number of synapses are decreased. Indeed, it has been proved that lesions of the hippocampus in humans prevent the acquisition of new episodic memories suggesting that hippocampus-dependent memory is mediated, at least in part, by hippocampal synaptic plasticity that is a prominent feature of hippocampal synapses of the neuronal network (Neves et al., 2008). Since the finding that the hippocampus plays a pivotal role in long-term memory consolidation (dogma, well established fact in the literature, described in the text books; e.g. Andersen et al., 2007; Byrne, 2008; Eichenbaum, 2002), many proposals have been made regarding its specific role. A prominent view of the mechanisms underlying consolidation of episodic memories involves fast formation (e.g., via Hebbian mechanisms) of strong associations between hippocampal sparse patterns of activity and distributed neocortical representations. Recent research on the primate prefrontal cortex discovered that the pyramidal cell circuits that generate the persistent firing underlying spatial working memory communicate through synapses on spines containing NMDARs with NR2B subunits (GluN2B) in the post-synaptic density. This contrasts with synapses in the hippocampus and primary visual cortex, where GluN2B receptors are both synaptic and extrasynaptic. Cholinergic stimulation of nicotinic α7 receptors within the glutamate synapse is necessary for NMDAR actions (Wang and Arnsten, 2015).

General role in biology:

Glutamatergic neurotransmission (NMDA, AMPA and KA receptors)

The network of glutamatergic neurons is heavily involved in long-term synaptic plasticity, the main process linked to learning and

memory. At the same time over-activation of these neurons (excitotoxicity) leads to neuronal cell death that can be mediated by increased levels of extracellular glutamate or a molecule that behaves as its analogue. Glutamate acts at a variety of ionotropic receptors, including AMPARs, kainate receptors, and NMDARs. The NMDARs have been of particular interest due to their unique properties. They require neuronal depolarization to relieve their Mg++ block, and are permeable to Ca++ that can initiate second-messenger signalling events, such as mediating neuroplasticity or negative feedback through Ca++-sensitive K+ channels. There have been extensive studies on the glutamate NMDAR and AMPAR mechanisms underlying long-term synaptic plasticity in the primary visual cortex and in CA1 neurons of the hippocampus (Liu et al., 2004; Cho et al., 2009; Lüscher and Malenka, 2012). Neuronal network function and long-term plasticity is also regulated by the levels of AMPAR expression as the number of AMPARs inserted into the post-synaptic density can mediate the degree of spine depolarization and thus the NMDAR opening. Synaptic plasticity in the mature visual cortex appears to be governed by GluN2A subunits, which have faster kinetics than GluN2B. GluN2B receptors are expressed in synapses early in development, but many move to extra-synaptic locations in the mature visual cortex and hippocampus (Goebel-Goody et al., 2009). The actions of NMDARs on the dorsolateral prefrontal cortex neuronal circuitry network underlying spatial working memory in primates and it mechanism is described in detail by Wang and Arnsten (2015). In the hippocampus, there is some evidence that long-term potentiation (LTP) is mediated by synaptic GluN2A, while long-term depression is mediated by extrasynaptic GluN2B receptors (Liu et al., 2004). Kainate receptors (KARs) also play an important role in neuronal network function. They play a major function in the pre-synaptic terminal, in particular in the hippocampus. Activation of kainate receptors in have been shown to regulate glutamate release (Jane et al., 2009) and to both depress and factilitate transmission in different synapses. Pre-synaptic kainate receptors in the hippocampus facilitate AMPA and NMDA receptor-mediated transmission at mossy fibre-CA3 synapses (Lauri et al, 2005). Activation of post-synaptic KARs facilitates activation of NMDARs as it has been described in the context of DomA exposure.

Role of other neurotransmitters

It is important to stress that other classical neurotransmitter systems also play an important role in learning and memory processes (Blokland 1996). The role of the most critical neurotransmitters has been evaluated in a meta-analysis based on studies of four behavioral tasks relevant for evaluation of rat cognitive functions such as Morris water maze, radial maze, passive avoidance, and spontaneous alternation (Myhrer, 2003). Calculation of impact factors (percentage of significant effects of chemical agents like agonists, antagonists, neurotoxins) showed that glutamate was ranking highest (93), followed by GABA (81), dopamine (81), acetylcholine (81), serotonin (55), and norepinephrine (48).

GABA-ergic receptors: indeed, presynaptic GABA B receptors mediate GABA-dependent inhibition of glutamate release, impacting plasticity of hippocampal synapses and hippocampus-dependent memory function (Vigot et al., 2006). A critical link between GABABR heterodimer conformational dynamics and local regulation of release probability at hippocampal synapses has been recently proved (Laviv et al., 2010).

5-Hydroxytrytamine (serotonin) type 3A receptors (5-HT3ARs), as the only ligand-gated ion channels in the serotonin receptor family, are known to regulate neuronal excitation and release of GABA in hippocampal interneurons, playing also an important role in glutamatergic synaptic plasticity. Deletion of the 5-HT3AR gene in transgenic mice abolished NMDAR-dependent long-term depression (LTD) induced by low-frequency stimulation (LFS) in hippocampal CA1 synapses in slices. In addition, 5-HT3ARs disruption inhibited AMPARs internalization, without altering basal surface levels of AMPARs. These observations revealed an important role of 5-HT3ARs in NMDAR-dependent long-term depression, which is critical for learning behaviours (Yu et al., 2014).

The cholinergic hypothesis claims that the decline in cognitive functions in dementia is predominantly related to a decrease in cholinergic neurotransmission. This hypothesis has led to great interest in the putative involvement of the cholinergic neurotransmission in learning and memory processes (Blokland 1996; Bracco et al., 2014).

Dopamine plays diverse roles in human behaviour and cognition but it is mainly involved in motivation, decision-making, reward processing, attention, working memory and learning (Steinberg and Janak, 2012; Labudda et al., 2010).

Noradrenaline is associated with memory processing as it induces lasting changes in the brain that could sustain memories over time (Gazarini et al., 2013). As confirmed later on its neurotransmission indeed strengthens memory-related synaptic plasticity such as long-term potentiation, allowing memories to be formed and maintained in a more intense and enduring manner, a notion particularly valid for those with emotional content (Joëls et al. 2011). Like other types of memory, an emotional memory has to be consolidated to allow its later retrieval. Accumulating evidence has indicated that noradrenaline acts during these gradual stages to fine-tune the strength and/or persistence of a memory (Guzmán-Ramos et al. 2012; Gazarini et al., 2013).

How it is Measured or Detected

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

Neuronal network activity is fundamental to brain function and now can be measured using in vitro and in vivo techniques such as:

1. Two-photon imaging of cell populations in vivo that are labelled with fluorescent calcium indicators. Two-photon imaging relies on fluorescence excitation and, in general, necessitates staining of cells with fluorescent dyes. Various staining methods have been developed for in vivo calcium measurements. Single cells can be filled with membrane-impermeable calcium indicators via intracellular recording electrodes or by single-cell electroporation. The basic aspects of in vivo calcium imaging and recent developments that allow evaluation of the neural circuits activity are described by Göbel et al., (2007a). With new imaging technology, scientists are now better able to visualize neural circuits connecting brain regions in humans. Advances in genetic engineering, microscopy, and computing are enabling scientists to begin to map the connections between individual nerve cells.

2. Optical detection of neuronal spikes both in vivo and in vitro. Assuming action potential (AP) as the only trigger of calcium influx, spike patterns are directly reflected in the trains of calcium transients. Each fluorescence trace is the convolution of the spike train with the single AP-evoked calcium transient plus added noise. The temporal resolution will be limited by the acquisition rate of the network scanning approach. In addition, the signal-to-noise ratio of fluorescence signals will be a decisive factor for the accuracy of the reconstruction.

3. Microelectrode array (MEA) recordings in primary cultures. Glutamate analogues effects on neuronal network activity can be assessed (Lantz et al., 2014) and neuronal spontaneous activity evaluation is already used for screening purposes (Valdivia et al., 2014).

4. To understand the function of a neural circuit, it is important to discriminate its sub-network components. This is possible through counterstaining of specific neuronal and glial cell types, especially in bulk loaded tissue where markers need not be calcium sensitive. In addition, transgenic mice with fluorescent protein expression in specific neuronal subsets, allow separation of functional signals into different neuronal subtypes (Göbel et al., 2007b).

5. Combined positron emission tomography (PET) and magnetic resonance imaging (MRI) is a new tool to study functional processes in the brain, including the response to a stimulus simultaneously using PET. Functional MRI (fMRI), is used to assesses at the same time fast vascular and oxygenation changes during activation. These results demonstrate the feasibility of combined PET-MRI for the simultaneous study of the brain at activation and rest, revealing comprehensive and complementary information to further decode brain function and brain networks (Wehrl et al., 2013).

6. Seed-based correlative analysis of [18F]fluorodeoxyglucose (FDG)-PET (FDG-PET) differences in images (resting state minus activation) is suitable to identify cerebral networks in rats. Using awake and freely moving animals enables functional network analysis of complex behavioral paradigms (Rohleder et al., 2015).

Although most experiments at present are carried out in anesthetized animals, several approaches for imaging in awake behaving animals have been devised that ultimately aim at directly correlating neuronal network dynamics with behaviour (Dombeck et al., 2007, Arenkiel et al., 2007). Finally, through expression of light-activated channel proteins, it might become possible in the future to not only read-out but also control neuronal networks in vivo (Garaschuk et al., 2006)since with the development of X-ray, CT, and MRI, deep neural networks involved in learning and memory processes can be studied in vivo (Cheng et al., .2014).

7. NMDAR overactivation-induced LTD that decrease number of spine density can be measured in vitro using GFP technology and by cofilin-F-actin quantification (Calabrese et al., 2014).

Current behavioural tests used for evaluating neural network function:

1. The Morris water maze: this test is developed to measure spatial orientation in rats The rat has to swim around the pool to search for a platform onto which he can escape from the water. In one condition, the platform is visible, rising 1 cm above the water surface. In a Second condition the rat has to learn to find the hidden platform provided it remains in the fixed position relative to distal room cues.

2. Radial maze: In the T-maze version of working memory, the animal has to remember only a single item for each trial. In the radial arm version of the working memory procedure rats have to learn multiple items.

3. Passive avoidance: fear-motivated avoidance tests are usually based on electric current as source of punishment.

4. Spontaneous alternation: spontaneous alternation is spatial alternation and represents a tendency to avoid stimulus re-exposure during exploratory behaviour. T-maze (simple or multiple), Y-maze, and radial maze are used to quantify an innate, unlearned response in rats.

These four behavioural tests are described in detail in the review by Myhrer (Myhrer et al., 2003).

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List of Adverse Outcomes in this AOP

Event: 1583: Sensory axonal peripheral neuropathy

Short Name: Sensory axonal peripheral neuropathy

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:279 - Microtubule interacting drugs lead to peripheral neuropathy	AdverseOutcome
Aop:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	AdverseOutcome
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Biological Context

Level of Biological Organization

Individual

Key Event Description

The peripheral nervous system (PNS) connects the central nervous system with peripheral tissues and can be divided into the visceral and the somatic nervous system.The somatic nervous system consists of sensory and motor neurons. While the motor neurons control the contraction of skeletal muscles, the sensory neurons receive information from joints, muscles and skin and send it to the CNS. Motor neuron cell bodies lie in the spinal cord but cell bodies of the sensory neurons are located in the dorsal root ganglia (DRG). In contrast to the central nervous system (CNS), the PNS is not protected by the blood-brain-barrier, the skull or the spinal column and is therefore highly vulnerable to toxicants and mechanical damage. [1] However, the PNS neurons exhibits a greater ability of regeneration compared to neurons of the CNS. [2]

Peripheral neuropathies mostly affect sensory neurons in a length-dependent manner and therefore are characterized by a stocking-and-glove distribution of the symptoms. Sensory symptoms occurring upon taxol treatment are for example numbness and paresthesia. [3-6] Reversibility of neuropathy upon discontinuation of treatment is reported. [7]

How it is Measured or Detected

- Retrospective study: Patients are interviewed after chemotherapy treatment and symptoms are assessed. [8, 9]

- Abnormal pin perception: Measurement of the distance in centimeters from the tip of the great toe or index finger to a level where normal pin sensation was sensed.

- Abnormalities in vibration and position sensation: Measurement at distal phalanx of the great toe or index finger. [9, 10]

- Reductions in strength of toe extensors, index finger abductors, arm abductors, hip flexors, foot dorsiflexors: Measurement by using the MRC scale and dynamometer [9, 10]

- Nerve conduction studies: Electrophysiological measurement of nerve conduction amplitudes and velocities in sensory neurons [9, 10]

- Quantitative sensory testing of vibratory threshold: Measurement of vibratory latency in seconds between the patient and the examiner in toes and fingers. [9, 10] Automated measurement using e.g. ‘Vibration 2’ (Physitemp Instruments Inc., Clifton NJ) which can be adjusted to different vibration amplitudes and records vibration units. [6]

- Quantitative sensory testing of thermal threshold: Using the Thermal Sensitivity Tester or NTE-2 (Physitemp Instruments Inc., Clifton NJ), the ability to discriminate small temperature differences at the index finger or the great toe can be quantified. [6, 11, 12]

- Sural nerve biopsy: A part of the sural nerve (some millimetres) is removed, fixed and investigated via light or electron microscopy. [13]

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Event: 351: Increased Mortality

Short Name: Increased Mortality

Key Event Component

Process	Object	Action
mortality		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	AdverseOutcome
Aop:96 - Axonal sodium channel modulation leading to acute mortality	AdverseOutcome
Aop:104 - Altered ion channel activity leading impaired heart function	AdverseOutcome
Aop:113 - Glutamate-gated chloride channel activation leading to acute mortality	AdverseOutcome
Aop:160 - Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality	AdverseOutcome
Aop:161 - Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality	AdverseOutcome
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:186 - unknown MIE leading to renal failure and mortality	AdverseOutcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	AdverseOutcome
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	AdverseOutcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome

AOP ID and Name	Event Type
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	AdverseOutcome
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	AdverseOutcome
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	AdverseOutcome
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	AdverseOutcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	AdverseOutcome
Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	AdverseOutcome
Aop:410 - GSK3beta inactivation leading to increased mortality via defects in developing inner ear	AdverseOutcome
Aop:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	AdverseOutcome

Biological Context

Level of Biological Organization

Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

All living things are susceptible to mortality.

Key Event Description

Increased mortality refers to an increase in the number of individuals dying in an experimental replicate group or in a population over a specific period of time.

How it is Measured or Detected

Mortality of animals is generally observed as cessation of the heart beat, breathing (gill or lung movement) and locomotory movements. Mortality is typically measured by observation. Depending on the size of the organism, instruments such as microscopes may be used. The reported metric is mostly the mortality rate: the number of deaths in a given area or period, or from a particular cause.

Depending on the species and the study setup, mortality can be measured:

- in the lab by recording mortality during exposure experiments
- in dedicated setups simulating a realistic situation such as mesocosms or drainable ponds for aquatic species
- in the field, for example by determining age structure after one capture, or by capture-mark-recapture efforts. The latter is a method commonly used in ecology to estimate an animal population's size where it is impractical to count every individual.

Regulatory Significance of the AO

Increased mortality is one of the most common regulatory assessment endpoints, along with reduced growth and reduced reproduction.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2653: AchE Inhibition leads to Activation of Cyp2E1

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	adjacent	Low	Moderate

Relationship: 2654: Activation of Cyp2E1 leads to Oxidative Stress in Brain

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	adjacent	Moderate	Moderate

Relationship: 2655: Oxidative Stress in Brain leads to N/A, Neurodegeneration

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	adjacent	High	High
Various neuronal effects induced by elavl3, sox10, and mbp	adjacent	Moderate	Moderate

Relationship: 647: N/A, Neurodegeneration leads to Decreased, Neuronal network function in adult brain

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	adjacent	Low	
Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	adjacent	High	High

Evidence Supporting Applicability of this Relationship

It has been shown at the neuromascular junction of D. melanogaster that quisqualate-type glutamate receptors are blocked by DomA (1 mM) (Lee et al., 2009). However, in crayfish (Procambarus clarkia) the same concentration of DomA has no effect in spike activity (Bierbower and Cooper, 2013).

Key Event Relationship Description

Neurodegeneration (retraction of dendrites or axons) or neuronal cell death decreases the number of synaptic connections affecting the neuronal network function (Seeley et al., 2009). Based on neuropathology (Braak and Braak, 1991), neuroimaging (Buckner et al., 2005 and Greicius et al., 2004), and evidence from transgenic animal models (Palop et al., 2007a), it is suggested that neurodegeneration leads to neural network dysfunction (Buckner et al., 2005 and Palop et al., 2006). In human spongiform encephalopathies, which cause rapidly progressive dementia, direct evidence supports disease propagation along affected trans-synaptic connections (Scott et al., 1992). For all other neurodegenerative diseases, there are limited human experimental data supporting the “network degeneration hypothesis.” It is demonstrated as a class-wide phenomenon, with major mechanistic significance, predicting that the spatial patterning of disease relates to some structural, metabolic, or physiological aspect of neural network biology dysfunction. Confirming the network degeneration hypothesis has clinical impact, stimulating development of new network-based diagnostic and disease-monitoring assays.

Evidence Supporting this KER

Biological Plausibility

Based on neuropathological findings and neuroimaging from patients suffering from neurodegeneration as well as from evidence derived by transgenic animal models of neurodegeneration, it has been suggested that neurodegeneration is related to neural network dysfunction (Palop et al., 2007b; Seeley et al., 2009). Neurodegeneration leads to impairment of retrograde axonal transport that prohibits the growth factor supply to long-range projection neurons, causing synapse loss, and post-synaptic dendrite retraction that leads to decreases of the neuronal network (Seeley et al., 2009).

Empirical Evidence

Include consideration of temporal concordance here

The effective concentration of DomA causing a decrease to 50% of control mean firing rate (MFR) values (EC50) in rat primary cultures (13-30 DIV) is 0.28 µM (Mack et al., 2014). Decrease of MFR has also been reported before by Hogberg et al. 2011, where mature cultures (28-35 DIV) have been exposed acutely to a wide range of concentrations of DomA. The concentration of 0.5 µM DomA significantly reduces MFR (77 %), the MBR (78 %)and the number of spikes per burst (71 %). Higher concentrations of DomA (1 and 2 µM) also significantly decrease the MFR, whereas concentrations up to 0.1 µM of DomA do not cause any effect on MFR (Hogberg et al., 2011). In primary rat cortical neurons (12-22 DIV), DomA (50 µM) has been reported to reduce MFR by more than 90% (McConnell et al., 2012).

Ten-minute exposure of rat hippocampal CA1 region slices to 400 nM DA causes depression of fEPSP (Qiu et al., 2009). After 1 h washout, fEPSP gradually has been gradually recovered. DomA-potentiated slices have shown also less tetanus-induced LTP compared with control slices when tested with either original stimulus or reset stimulus (Qiu et al., 2009). In addition, prolonged application of 400 nM DA reversibly depresses CA1 fEPSP and impairs the subsequent development of tetanus LTP (Qiu et al., 2009).

Gap of knowledge: there are no experiments to support such a KE relationship after exposure to GLF.

Uncertainties and Inconsistencies

Administration of high dose DomA (4.4 mg/kg) to adult male Sprague-Dawley rats causes elevation of electrocorticogram (ECoG) beginning 30 min post injection, whereas at a lower dose (2.2 mg/kg) ECoG becomes elevated after 110 min (Binienda et al., 2011).

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Relationship: 2656: Decreased, Neuronal network function in adult brain leads to Sensory axonal peripheral neuropathy

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	adjacent	Moderate	Low

List of Non Adjacent Key Event Relationships

Relationship: 2657: Sensory axonal peripheral neuropathy leads to Increased Mortality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	non-adjacent	Low	Low