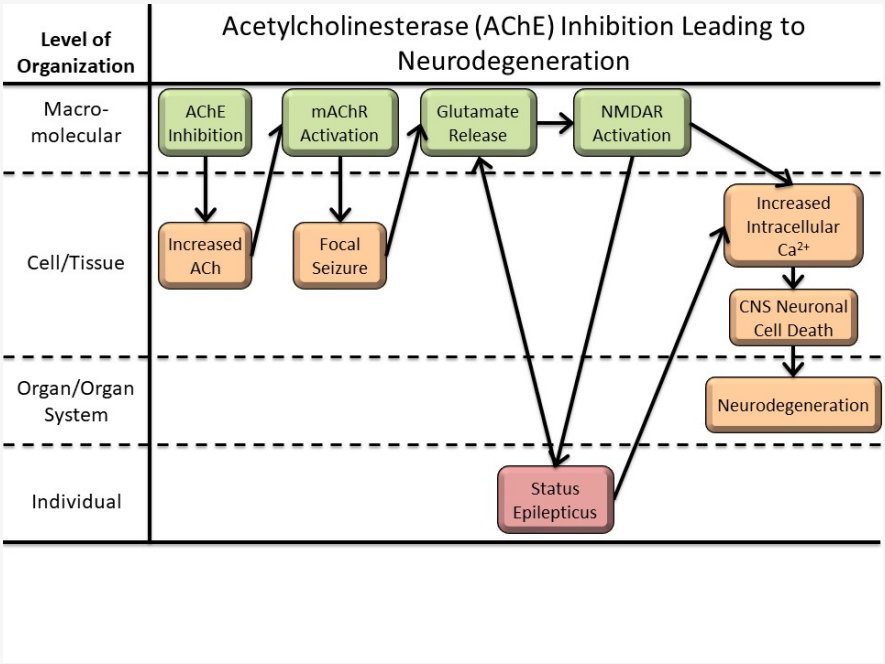
AOP ID and Title:

AOP 281: Acetylcholinesterase Inhibition Leading to Neurodegeneration
Short Title: AChE Inhibition Leading to Neurodegeneration

Graphical Representation



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Abstract

The enzyme acetylcholinesterase (AChE) hydrolyzes acetylcholine (ACh) in order to eliminate it from the body. When AChE is inhibited ACh levels increase. An excess of ACh at cholinergic synapses overstimulates both muscarinic- and nicotinic- receptors (1,2). These receptors are found in most organs in the body, thus the effects of AChE inhibition can result in multiple adverse outcomes affecting a wide variety of functions (1). This AOP focuses upon an acute outcome of neurodegeneration due to AChE inhibition specifically through calcium dysregulation as that has been identified as central to the development of the most severe phenotype caused by acute organophosphate poisoning (3).

1. United States., Environmental Protection Agency., Office of Pesticide Programs. (2000). The Use of Data on Cholinesterase Inhibition for Risk Assessments of Organophosphorous and Carbamate Pesticides. https://www.epa.gov/sites/production/files/2015-07/documents/cholin.pdf accessed Nov. 2018.

2. Quick, M. W., & Lester, R. A. J. (2002). Journal of Neurobiology, 53(4), 457-478. doi:10.1002/neu.10109.

3. Faria et al. (2015). Scientific Reports, 5. doi:10.1038/srep15591.

Background

Epidemiological studies concerning OP pesticides estimated approximately 3 million cases of acute severe poisoning, as well as 300,000 deaths annually. Most of those deaths occur in developing countries of the Asia-Pacific region (Bertolote et al., 2006). These OP compounds can also be used as chemical warfare nerve

agents. The improper use of OP chemicals has tragic consequences such as neurodegeneration, brain damage, and death underscoring the need for safety measures that protect both human health and the environment.

Bertolote, J. M., Fleischmann, A., Eddleston, M. & Gunnell, D. 2006. Deaths from pesticide poisoning: A global response. *British Journal of Psychiatry,* 189**,** 201-203. DOI: 10.1192/bjp.bp.105.020834.

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	10	Acetylcholine accumulation in synapses	ACh Synaptic Accumulation
3	KE	1602	Activation, Muscarinic Acetylcholine Receptors	Activation, Muscarinic Acetylcholine Receptors
4	KE	1623	Occurrence, Focal Seizure	Occurrence, Focal Seizure
5	KE	1350	Increased, glutamate	Increased, glutamate
6	KE	388	Overactivation, NMDARs	Overactivation, NMDARs
8	KE	1788	Status epilepticus	Status epilepticus
7	KE	389	Increased, Intracellular Calcium overload	Increased, Intracellular Calcium overload
9	KE	55	Cell injury/death	Cell injury/death
10	AO	352	N/A, Neurodegeneration	N/A, Neurodegeneration

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Acetylcholinesterase (AchE) Inhibition	adjacent	Acetylcholine accumulation in synapses	High	Moderate
Acetylcholine accumulation in synapses	adjacent	Activation, Muscarinic Acetylcholine Receptors	Moderate	High
Activation, Muscarinic Acetylcholine Receptors	adjacent	Occurrence, Focal Seizure	Moderate	Low
Occurrence, Focal Seizure	adjacent	Increased, glutamate	Moderate	Low
Increased, glutamate	adjacent	Overactivation, NMDARs	Moderate	High
Overactivation, NMDARs	adjacent	Status epilepticus	Moderate	Low
Status epilepticus	adjacent	Increased, glutamate	Moderate	Low
Overactivation, NMDARs	adjacent	Increased, Intracellular Calcium overload	High	Moderate
Status epilepticus	adjacent	Increased, Intracellular Calcium overload	High	Low
Increased, Intracellular Calcium overload	adjacent	Cell injury/death	High	Low
Cell injury/death	adjacent	N/A, Neurodegeneration	High	Low

Overall Assessment of the AOP

Domain of Applicability

Sex: The AOP is not sex-specific

Life stages: the AOP is relevant to all life stages. Immature or developing populations may be more sensitive due to their increased susceptibility to seizures and developing cholinergic systems.

Taxonomic: given that both cholinergic and glutamatergic systems are highly conserved among vertebrates, this AOP is likely to be applicable to all vertebrates.

Essentiality of the Key Events

All KEs in AOP 281 rank *high* for essentiality. The provided studies demonstrate direct evidence and include experiments involving inhibition of AChE through the application of various inhibitors, gene-knockout experiments, receptor antagonist studies, and anticonvulsant treatments which are shown to result in the reduction of neurodegeneration.

- AChE Inhibition (MIE) evidence is *high*. This is supported by several studies that measured increases in ACh after inhibition of AChE by a variety of inhibitors (Del Pino et al., 2017, Karanth et al., 2007, Kim et al., 2003, Kosasa et al., 1999, Ray et al., 2009). Additionally, researchers have demonstrated that pretreatment with a combination of reversible AChE inhibitors, nicotinic and mAChR receptor antagonists prior to exposure to soman resulted in a significantly higher survival rate and overall reduced brain ACh levels compared to controls (Harris et al., 1980).
- ACh accumulation in synapses (KE 1) evidence is *high*. Blocking the effects of ACh with atropine, an mAChR antagonist, was demonstrated to significantly reduce the pathological effects and neurodegeneration associated with soman intoxication (McDonough et al., 1989).
- Activation of mAChRs (KE 2) evidence is *high*. M1-mAChR deficient mice through gene-knockout studies were shown to be resistant to seizures induced by pilocarpine, an mAChR agonist (Hamilton et al., 1997).
- Occurrence of Focal Seizure (KE 3) evidence is *high*. Treatment with diazepam, a $GABA_A$ receptor agonist and known anticonvulsant, both prevented seizures and resulted in significantly reduced brain pathology (McDonough et al., 1989).
- Increased Glutamate (KE 4) evidence is *high*. Application of 500 μM of glutamate showed in reduction in neuron survival, however if NMDA antagonist MK-801 was used in conjunction with glutamate, neuron survival returned to control levels (Michaels and Rothman, 1990). Other *in vivo* experiments using MK-801 or ketamine demonstrated a reduction in seizure activity and reduced neurodegeneration (Borris et al., 2000, Braitman and Sparenborg, 1989, Sparenborg et al., 1992).
- Overactivation of NMDARs (KE 5) evidence is *high*. Multiple experiments using ketamine and MK-801, both NMDA receptor antagonists, have been demonstrated to terminate or reduce both seizure activity and neurodegeneration (Borris et al., 2000, Braitman and Sparenborg, 1989, Sparenborg et al., 1992).
- Increased Intracellular Calcium Overload (KE 6) evidence is *high*. Calcium chelation in zebrafish models of organophosphate exposure significantly reduced neurodegeneration (Faria et al., 2015). Additionally, Deshpande et al. (2008) demonstrated that cell death could be significantly reduced given a low extracellular calcium solution in an *in vitro* model of SE in rat hippocampal neurons.
- Status Epilepticus (KE 7) evidence follows that of KE 3 and is considered *high*. Anticonvulsant treatment using diazepam was demonstrated to significantly reduce neurodegeneration (McDonough et al., 1989).
- Cell Injury/Death (KE 8) evidence is considered *high*. Cell death in the context of the brain is considered a form of neurodegeneration (Przedborski et al., 2003). Therefore, prevention of cell death directly results in the prevention of the adverse outcome.

Weight of Evidence Summary

Biological plausibility: Biological plausibility refers to the structural or functional relationship between the key events based on our fundamental understanding of "normal biology". The evidence for biological plausibility throughout this AOP from inhibition of AChE to neurodegeneration is high. It is well understood that inhibition of AChE is followed by an accumulation of A**C**h, which subsequently leads to activation of muscarinic acetylcholine receptors and focal seizures. The seizures then lead to increase**d** glutamate, which binds to and overactivates NMDARs. Following that step, we find the highest biological uncertainty in the pathway, with moderate biological plausibility (from overactivation of NMDARs leading to status epilepticus to increased glutamate). The rest of the pathway is considered of high biological plausibility all the way to neurodegeneration.

Concordance of dose-response relationships: Dose response concordance considers the degree to which upstream events are shown to occur at test concentrations equal to or lower than those that cause significant effects on downstream key events, the underlying assumption being that all KEs can be measured with equal precision. There is a significant amount of quantitative data providing dose and temporal concordance for multiple species between AChE inhibitors and the resulting percent AChE inhibition and ACh concentration (Kosasa et al., 1999). Dose-response relationships have been well stablished by showing that AChE inhibition resulted in the progressive accumulation of extracellular ACh. Furthermore, the relationship between increased intracellular calcium and cell death through dose and temporal concordance has also been demonstrated. Additionally, Faria et al. (2015) demonstrated a dose-response relationship between increasing doses of the organophosphate chlorpyrifos-oxon and the prevalence of a severe phenotype marked by measurably increased necrosis.

Temporal concordance: Temporal concordance refers to the degree to which the data support the hypothesized sequence of the key events; i.e., the effect on KE1 is observed before the effect on KE2, which is observed before the effect on KE3 and so on. Temporal concordance has been shown between seizure activity and increasing levels of glutamate (KE4 and KE7). Furthermore, temporal concordance has also been established between status epilepticus and increased intracellular calcium in rats. The relationship between increased intracellular calcium and cell death through dose and temporal concordance has also been demonstrated.

Consistency: We are not aware of cases where the whole chain of key events described was observed without also observing a significant impact on neurodegeneration. Nevertheless, the final adverse outcome is not specific to this AOP. Many of the key events included in this AOP overlap with AOPs linking other molecular initiating events to other adverse outcomes.

Uncertainties, inconsistencies, and data gaps: The current main uncertainties within this AOP are related to seizures and the location of AChE inhibition. Even though it is well known that there are two phases of seizure activity driven by cholinergic and glutamatergic mechanisms, the transition between these phases is not well understood. Additionally, the levels of increasing glutamate post-AChE inhibition appear to be dependent on the location of inhibition as well as stressor specific.

Quantitative Consideration

At present, the quantitative understanding of this AOP varies by level of biological organization. While the initial KEs leading to activation of muscarinic acetylcholine receptors have a high level of quantitative understanding, following KEs leading all the way to the Adverse Outcome (Cell Injury / Death Leading to Neurodegeneration) have a much lower quantitative understanding. The exception would be KE5 (Increased Glutamate leading to Overactivation of NMDARs), that has multiple kinetic models available to evaluate quantitative relationships. Overall, better quantitative relationships need to be developed to be able to quantitively and effectively predict the adverse outcome.

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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

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

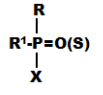
- Organophosphate and carbamate insecticides are prototypical AChE inhibitors. The OP and carbamate pesticides were synthesized specifically to act as inhibitors of AChE, with OPs developed from early nerve agents (e.g., sarin) and carbamate pesticides based on the natural plant alkaloid physostigmine (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

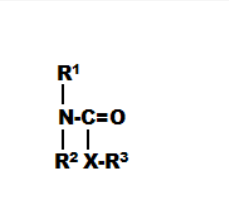
Evidence exists that immature life stages in mammals and birds may be more sensitive to organophosphate pesticides (see Grue et al., 1997; Grue et al., 1983; Grue et.al; 1981). It has been suggested that this may be related to the amount of pesticide ingested in relation to body size (Ludke et al, 1975), 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: 10: Acetylcholine accumulation in synapses

Short Name: ACh Synaptic Accumulation

Key Event Component

Process	Object	Action
	acetylcholine	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	KeyEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	KeyEvent
Aop:405 - Organo-Phosphate Chemicals induced inhibition of AChE leading to impaired cognitive function	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebra fish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

- Acetylcholine and cholinergic receptors are found in invertebrate and vertebrate species. Specific examples from the literature documenting acetylcholine accumulation include: Penaeid prawn exposed to sublethal exposure of methylparathion and malathion showed significantly increased ACh levels, in nervous tissue (Reddy 1990).
- Brain tissue of tadpoles exposed to single sublethal concentrations methyl parathion for 24 h showed an increase in acetylcholine levels (Nayeemunnisa and Yasmeen 1986).
- Acute (48h) sublethal exposure to methyl parathion resulted in increased AChE levels in brain tissue in fish (Oreochromis mossambicus) (Rao and Rao, 1984). Researchers found a significant increase in acetylcholine at all time points measured (12-48hr) with acetylcholine levels increasing from 33-83% as compared to controls over the same time span.
- A study of male quail (Coturnix japonica) exposed to lethal concentrations of two OP pesticides (i.e., DDVP or fenitrothion), found significant increases in total and free acetylcholine (Kobayashi et al., 1983).
- Mice singly injected with propoxur displayed changes in cholinergic parameters in the brain: increased brain ACh content, decreased AChE activity, and high-affinity choline uptake into synaptosomes (Kobayashi 1988).
- AChE levels and acetylcholine synthesis in rat striatum were compared in controls and animals exposed to 0.3 and 1 times the maximum tolerated dose. Acetylcholine was present in significantly less concentrations than in the adult rats (Karanth, 2003).

Key Event Description

- Acetylcholine is a neurotransmitter that is stored in nerve endings at cholinergic synapses in the central and peripheral nervous systems (Soreq and Seidman, 2001; Lushington 2006).
- Acetylcholine can bind multiple types of nicotinic and muscarinic receptors. The downstream consequences of those events are tissue and receptor-specific.
- Acetylcholine is released into the synaptic cleft when stimulation of the nerve occurs, and then binds to a receptor protein; either muscarinic (metabotropic) or nicotinic (ionotropic). The binding to the receptor results in changes in the flow of ions across the cell, thereby signaling activity (Fukuto 1990; Mileson et al 1998; Soreq and Seidman 2001; Lushington 2006).
    - Inhibition of acetylcholine binding at the serine site via AChE inhibition results in an accumulation of acetylcholine in synapses associated with muscarinic and nicotinic receptors, resulting in unregulated excitation at neuromuscular junctions of skeletal muscle; pre-ganglionic neurotransmitters and post-ganglionic nerve endings of the autonomic nervous system; and neurotransmitters in the brain or central nervous system (CNS).

How it is Measured or Detected

- Several techniques are available to measure acetylcholine levels, including the Hestrin method (Augustinsson 1957, Hestrin 1949, Stone 1955), molecular probes or assays, microdialysis techniques (Zapata, 2009, Russom, 2014) or by liquid chromatography - tandem mass spectrometer LC-MS/MS (Gómez-Canela et al., 2017).
- Hestrin's method involves a colorimetric measurement of esterase activity. The rate of hydrolysis of acetylcholine with hydroxylamine to form hydroxamic acid is measured to determine the amount of acetylcholine:

$$RCOOR' + H2NOH \rightarrow RCONHOH + R'OH$$

This method is performed at alkaline pH in water and is applicable over a wide range of ester concentrations (Hestrin 1949).

- Hydrolysis of acetylcholine by acetylcholinesterase in the synaptic cleft is fast, so concentration in the extracellular fluid is low (0.1-6 nM). Brain microdialysate studies quantify nanomolar concentrations of acetylcholine in extracellular fluid using chromatographic mass spectrometric techniques (Nirogi 2009). Choice of analytical method should provide detection limits below the lowest concentration expected in the dialysate and requiring the smallest sample volume. High-pressure liquid chromatography coupled to electrochemical detection (HPLC-EC) is based on enzymatic conversion of acetylcholine into choline and acetate by acetylcholinesterase, and subsequent oxidation of choline by choline oxidase to betaine and hydrogen peroxide, which can be oxidized on a platinum electrode. This method permits detection of dialysate acetylcholine concentrations in the 5-10 nM range (Zapata, 2009). Other microdialysis techniques for quantification of acetylcholine are liquid chromatography mass spectrometry (Nirogi 2009) and pyrolysis-gas chromatography (Szilagyi 1968).

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Event: 1602: Activation, Muscarinic Acetylcholine Receptors

Short Name: Activation, Muscarinic Acetylcholine Receptors

Key Event Component

Process	Object	Action
G-protein coupled acetylcholine receptor binding	muscarinic acetylcholine receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability

Term	Scientific Term	Evidence	Links

Life Stage	Evidence
Embryo	Moderate
Juvenile	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxa:

mAChRs are found in most vertebrates, many of the studies cited are conducted using zebrafish and mice. Zebrafish are frequently used for high-throughput assays as they have well-conserved neurotransmitter structures, including acetylcholine transmitters (Garcia et al., 2016). This can provide valuable data regarding the activation of mAChRs in mammalian systems. Knockout mice also help to elucidate the functions of specific mAChR subtypes (Gainetdinov and Caron, 1999).

Life stage:

mAChRs signal neurons throughout all life stages (Miller and Yeh, 2016). They do not only affect individuals during developmental stages, but there have been some studies conducted specifically on the developmental effects of chemicals that affect acetylcholine signaling (Burke et al., 2017). Most of the whole animal experimental data are from younger specimens, but there have also been experiments on adult individuals (Fitzgerald and Costa, 1993).

Sex:

mAChRs are found in both males and females, with similar functions (Burke et al., 2017).

Key Event Description

Muscarinic acetylcholine receptors (mAChRs) are G-protein-coupled receptors (GPCRs) with five different subtypes (M1, M2, M3, M4, and M5). GPCRs are transmembrane receptors that detect extracellular signals and activate internal pathways which modulate a variety of processes such as locomotion, learning and memory, thermoregulation and epileptic seizures (Gainetdinov and Caron, 1999). Subtypes M1, M3, and M5 are Gq- coupled receptors that activate phospholipase C enzyme resulting in two secondary messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Subtypes M2 and M4 are inhibitory and signal using the G_i pathway (Haga, 2013). G_i protein activation inhibits adenylyl cyclase, and reduces the conversion of ATP to cAMP (Jett and Lein, 2011).

In its resting state, the mAChR G-protein subunits (alpha, beta and gamma) are clustered together and the alpha subunit is bound to GDP. Once a ligand binds to an mAChR, the receptor undergoes a conformation change that allows the alpha subunit to exchange its bound GDP with GTP, then the alpha subunit dissociates from the beta and gamma subunits. Once the alpha subunit is free of the beta and gamma subunits, it moves along the cell membrane to affect its target enzyme, which typically sends out secondary messenger signals (Kandel et al., 2013)

How it is Measured or Detected

Most studies investigating the function of mAChRs involve blocking signaling from these receptors through use of selective antagonists like atropine or scopolamine, or the use of gene targeted knockout specimens (Bymaster et al. 2003; Faria et al. 2017). The distribution and density of mAChRs can be measured using radiolabeled agonists that bind to the mAChR binding site. The receptor activity can be measured by detecting secondary-messengers regulated by the G-protein.

- Use mAChR agonist [^{3}H] quinuclidinyl benzilate (QNB) to label mAChRs (all subtypes; see Fonnum and Sterri (2011) and measure binding levels as described by Fitzgerald and Costa (1993) and Gazit et al. (1979)
- Determination of the relative levels of specific mAChR subtypes in tissues has been found through the use of subtype-specific antisera as described by Dörje et al. (1991)
- Kinetic measurements of DAG production and IP3 release can be obtained through fluorescent reporters as in Falkenburger et al. (2013) and Dickson et al. (2013).
- Changes in the activity and quantity of cAMP and the cAMP-dependent protein kinases can serve as an indicator of the activity of mAChRs bound to Gi-proteins (M2 and M4). cAMP content can be determined using a radioimmunoassay (RIA) kit (Heikkilä et al., 1991).
- Adenylyl cyclase activity can be determined through an assay as described by Salomon et al. (1974) and used by Raheja and Dip Gill (2007).

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Event: 1623: Occurrence, Focal Seizure

Short Name: Occurrence, Focal Seizure

Key Event Component

Process	Object	Action
EEG with focal epileptiform discharges	neuron	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI

Term	Scientific Term	Evidence	Links
guinea pig	Cavia porcellus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxa

Seizures have been observed and studied in many different species including vertebrate and invertebrates. Listed species above are specifically referenced in the cited sources.

Age

There is evidence indicating that in developing rat brains GABAergic activity might be excitatory, not inhibitory (Li and Xu, 2008). Increased sensitivity shown by younger individuals to some substances that induce seizures may possibly be affected by this phenomenon (Miller, 2015).

Sex

Both males and females can develop focal seizures, with some possible differences in sensitivity to certain forms of epileptic activity (Belelli et al., 1990). Despite some differences the effect of the key event is conserved for both sexes.

Key Event Description

This key event is characterized as the start of synchronized neural signaling in a specific group of neurons. It is possible that when the ratio between excitatory (glutamatergic) over inhibitory (GABAergic) currents in brain tissue increases past the threshold of the network , seizure starts to occur (Miller, 2015). The initial occurrence of epileptiform activity, in specific regions of the brain, can begin a signaling cascade leading to seizure spread throughout the brain (i.e., secondary generalization leading to status epilepticus) (Kandel et al., 2013).

Acetylcholinesterase inhibition induced seizure

For the signaling cascade caused by acetylcholinesterase inhibition to continue to propagate, some studies suggest that stimulation specifically in the basolateral amygdala plays a key role in the development of seizure activity (McDonough Jr and Shih, 1997). Other studies indicate that the piriform cortex as well as the hippocampus also play a role in seizure development caused by nerve agents (Myhrer, 2007).

How it is Measured or Detected

- An electrocorticogram record can be used to measure brain activity to monitor seizure development (Braitman and Sparenborg, 1989).
- Brain electroencephalographic (EEG) activity can also record the development of the seizure (Acon-Chen et al., 2016; Kandel et al., 2013).
- Whole cell recordings of spontaneous inhibitory postsynaptic currents and excitatory postsynaptic currents have also been used to study the initial seizures occurring from exposure to organophosphates (Miller, 2015).

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Event: 1350: Increased, glutamate

Short Name: Increased, glutamate

Key Event Component

Process	Object	Action
synaptic transmission, glutamatergic	L-glutamate(1-)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:215 - Molecular events lead to epilepsy	KeyEvent
Aop:230 - presynaptic neuron 1 activation to epilepsy	KeyEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:490 - Co-activation of IP3R and RyR leads to economic burden through reduced IQ and non-cholinergic mechanisms	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
neuron

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	High

Taxa:

Zebrafish neurotransmitter systems, including glutamate, are being used more for investigating chemical toxicity (Horzmann and Freeman 2016). Some cited sources above have data from rat experiments.

Life Stage:

Glutamate is functional throughout all life stages. Liu et al. (1996) suggests that immature rat brains show less glutamate-induced neurotoxicity than adult brains.

Sex:

Glutamate and glutamate receptors have been studied in both males and females, with similar functionality (Jafarian et al. 2019).

Key Event Description

Glutamate (Glu) release into the synaptic cleft is primarily caused by excitatory glutamatergic neurons, however there is evidence showing astrocytes releasing glutamate through a calcium-dependent process. A mechanism explaining how astrocytes release glutamate is not well defined, but it could be released through exocytosis(Nedergaard et al. 2002). Glutamate is the main excitatory transmitter in the brain and spinal cord, where it activates both ionotropic and metabotropic receptors. There are 3 main ionotropic receptor classifications, AMPA, Kainate, and NMDA receptors, which are always excitatory (Kandel et al. 2013: 213). Excessive extracellular glutamate release overactivates these signaling pathways, and propagates the excitotoxicity caused by some nerve agents (McDonough and Shih 1997).

How it is Measured or Detected

- Glutamate uptake by astrocytes and synaptic cleft concentration can be measured using liquid scintillation spectrometry and radiolabeled glutamate (H^3 glutamate) (Lallement et al. 1991). Liquid scintillation spectrometry counts the activity of a radioactive sample by mixing the glutamate with a liquid scintillator (a material that fluorescens) and count photon emissions.

- Another mechanism to measure the glutamate concentration in the synaptic cleft is by microdialysis sampling. This mechanism is inexpensive and easy to use. When microdialysis is paired with other analytical methods such as High-Pressure Liquid Chromatography (HPLC), there is a higher instrumental selectivity and sensitivity (Watson et al. 2006).

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Event: 388: Overactivation, NMDARs

Short Name: Overactivation, NMDARs

Key Event Component

Process	Object	Action
NMDA glutamate receptor activity	NMDA selective glutamate receptor complex	increased

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:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	MolecularInitiatingEvent
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

neuron

Domain of Applicability

Taxonomic Applicability

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

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
zebrafish	Danio rerio	High	NCBI

It is important to note that in invertebrates the glutamatergic synaptic transmission has an inhibitory and not an excitatory role like in vertebrates. This type of neurotransmission is mediated by glutamate-gated chloride channels that are members of the 'cys-loop' ligand-gated anion channel superfamily found only in invertebrates. The subunits of glutamate-activated chloride channel have been isolated from C. elegans and from Drosophila (Blanke and VanDongen, 2009).

Key Event Description

Biological state: Please see MIE NMDARs, Binding of antagonist

Biological compartments: Please see MIE NMDARs, Binding of antagonist

General role in biology: Please see MIE NMDARs, Binding of antagonist

The above chapters belong to the AOP entitled: *Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities* since the general characteristic of the NMDA receptor biology is the same for both AOPs.

Additional text, specific for this AOP:

At resting membrane potentials, NMDA receptors are inactive. Depending on the specific impulse train received, the NMDA receptor activation triggers long term potentiation (LTP) or long-term depression (LTD) (Malenka and Bear, 2004; Luscher and Malenka, 2012). LTP (the opposing process to LTD) is the long-lasting increase of synaptic strength. For LTP induction both pre- and postsynaptic neurons need to be active at the same time because the postsynaptic neuron must be depolarized when glutamate is released from the presynaptic bouton to fully relieve the Mg2+ block of NMDARs that prevents ion flows through it. Sustained activation of AMPA or KA receptors by, for instance, a train of impulses arriving at a pre-synaptic terminal, depolarizes the post-synaptic cell, releasing Mg2+ inhibition and thus allowing NMDA receptor activation. Unlike GluA2-containing AMPA receptors, NMDA receptors are permeable to calcium ions as well as being permeable to other ions. Thus NMDA receptor activation leads to a calcium influx into the post-synaptic cells, a signal that is instrumental in the activation of a number of signalling cascades (*Calcium-dependent processes are describe in Key Event Calcium influx, increased*). Postsynaptic Ca2+ signals of different amplitudes and durations are able to induce either LPT or LTD.

Conversely to LTP, LTD is induced by repeated activation of the presynaptic neuron at low frequencies without postsynaptic activity (Luscher and Malenka, 2012). Therefore, under physiological conditions LTD is one of several processes that serves to selectively weaken specific synapses in order to make constructive use of synaptic strengthening caused by LTP. This is necessary because, if allowed to continue increasing in strength, synapses would ultimately reach a ceiling level of efficiency, which would inhibit the encoding of new information (Purves, 2008).

LTD is an activity-dependent reduction in the efficacy of neuronal synapses lasting hours or longer following a long patterned stimulus. It has also been found to occur in different types of neurons however, the most common neurotransmitter involved in LTD is L-glutamate that acts on the NMDARs, AMPAR, KARs and metabotropic glutamate receptors (mGluRs). It can result from strong synaptic stimulation (as occurs e.g. in the cerebellar Purkinje cells) or from persistent weak synaptic stimulation (as in the hippocampus) resulting mainly from a decrease in postsynaptic AMPA receptor density, although a decrease in presynaptic neurotransmitter release may also play a role. Moreover, cerebellar LTD has been hypothesized to be important for motor learning and hippocampal LTD may be important for the clearing of old memory traces (Nicholls et al., 2008; Mallere et al., 2010). The main molecular mechanism underlying-LTD is the phosphorylation of AMPA glutamate receptors and their synaptic elimination (Ogasawara et al., 2008).

It is now commonly understood in the field of spine morphology that long lasting NMDAR-dependent LTD causes dendritic spine shrinkage, reduces number of synaptic AMPA receptors (Calabrese et al., 2014), possibly leading to synaptic dysfunction, contributing to decreased neuronal network function and impairment of learning and memory processes.

Additional text, specific for the AOP "Acetylcholinesterase inhibition leading to neurodegeneration":

Seizures caused by cholinesterase dependent mechanisms result in an excess of glutamate release that activates the NMDA receptors. As a result, intracellular Ca2+ levels at the postsynaptic neuron can overload the calcium-control mechanisms, activating without control all the calcium-dependent enzymes (proteases, lipases…) (Deshpande et al., 2014; Garcia-Reyero et al., 2016). In cases of strong acetylcholinesterase inhibition of the CNS, the NMDAR overactivation initiated by cholinergic mechanisms can result, after the initial seizure activity (focal seizure), in the development of status epilepticus. This key event separates the initial toxicity, driven by cholinergic activity, from the secondary toxicity, which is cholinergic independent (McDonough and Shih, 1997).

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?

No OECD methods are available to measure the activation state of NMDA receptors.

The measurement of the activation or the inhibition of NMDA receptors is done indirectly by recording the individual ion channels that are selective to Na+, K+ and Ca2+ by the patch clamp technique. This method relies on lack of measurable ion flux when NMDA ion channel is closed, whereas constant channel specific conductance is recorded at the open state of the receptor (Blanke and VanDongen, 2009). Furthermore, this method is based on the prediction that activation or inhibition of an ion channel results from an increase in the probability of being in the open or closed state, respectively (Ogdon and Stanfield, 2009; Zhao et al., 2009).

The whole-cell patch clamp recording techniques have also been used to study synaptically-evoked NMDA receptor-mediated excitatory or inhibitory postsynaptic currents (EPSCs and IPSCs, respectively) in brain slices and neuronal cells, allowing the evaluation of the activated or inhibited state of the receptor.

Microelectrode array (MEA) recordings are used to measure mainly spontaneous network activity of cultured neurons (Keefer et al., 2001, Gramowski et al., 2000 and Gopal, 2003; Johnstone et al., 2010). However, using specific agonists and antagonists of a receptor, including NMDAR, MEA technology can be used to measure evoked activity, including glutamatergic receptor function (Lantz et al., 2014). For example it has been shown that

MEA-coupled neuronal cortical networks are very sensitive to pharmacological manipulation of the excitatory ionotropic glutamatergic transmission (Frega et al., 2012). MEAs can also be applied in higher throughput platforms to facilitate screening of numerous chemical compounds (McConnell et al., 2012).

Excessive excitability can be also measured directly by evaluating the level of the extracellular glutamate using the enzyme-based microelectrode arrays. This technology is capable of detecting glutamate in vivo, to assess the effectiveness of hyperexcitability modulators on glutamate release in brain slices. Using glutamate oxidase coated ceramic MEAs coupled with constant voltage amperometry, it is possible to measure resting glutamate levels and synaptic overflow of glutamate after K(+) stimulation in brain slices (Quintero et al., 2011).

Neuronal network function can be also measured using optical detection of neuronal spikes both in vivo and in vitro (Wilt et al., 2013).

Drebrin immunocytochemistry: drebrin, a major actin-filament-binding protein localized in mature dendritic spines is a target of calpain mediated proteolysis under excitotoxic conditions induced by the overactivation of NMDARs. In cultured rodent neurons, degradation of drebrin was confirmed by the detection of proteolytic fragments, as well as a reduction in the amount of full-length drebrin. The NMDA-induced degradation of drebrin in mature neurons occurres concomitantly with a loss of f-actin. Biochemical analyses using purified drebrin and calpain revealed that calpain degraded drebrin directly in vitro. These findings suggest that calpain-mediated degradation of drebrin is mediated by excitotoxicity, regardless of whether they are acute or chronic. Drebrin (A and E) regulates the synaptic clustering of NMDARs. Therefore, degradation of drebrin can be used as a readout for excitotoxicity induced by NMDAR overactivation. Degradation of drebrin can be evaluated quantitatively by Western blot analysis (mRNA evel) or by immunocytochemistry (at protein level) (Chimura et al., 2015: Sekino et al., 20069.

NMDAR overactivation-induced long lasting LTD can be measured by the dendritic spine shrinkage by quantification of cofilin and phospho-cofilin in neurons expressing eGFP and combined with immunocytochemical techniques (Calabrese et al., 2014).

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Event: 1788: Status epilepticus

Short Name: Status epilepticus

Key Event Component

Process	Object	Action
secondary generalized seizure	brain	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Moderate	NCBI
guinea pig	Cavia porcellus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

See KE Occurrence, Focal Seizure.

Key Event Description

Focal seizures occur when a small group of neurons start synchronized neural signaling (See KE Occurrence, Focal Seizure). Once started, focal seizures can spread to the entire brain through various axonal pathways. GABA-ergic interneurons help inhibit seizure spread from the seizure focus forming an inhibitory region. If the activity in the focus is intense enough that inhibitory region breaks down and the seizure spreads (Kandel et al., 2013). Once the epileptiform activity has expanded to other areas in the brain, i.e., once both hemispheres of the brain are involved for approximately 5 minutes, the focal seizure has been secondarily generalized (status epilepticus) (Lowenstein and Alldredge, 1998).

Acetylcholinesterase inhibition induced seizure

In the case of acetylcholinesterase inhibition, status epilepticus has been seen to be regulated through NMDAR activation and increasing intracellular Ca2+, which is distinct from the initial focal seizure through mAChRs (Acon-Chen et al., 2016). Anticholinergic drugs (atropine, 2-PAM…) are ineffective if administrated after seizure generalization, whereas NMDAR antagonists (memantine, MK-801…) can still be effective 35 minutes after exposure (Lallement et al., 1999; McDonough and Shih, 1997).

How it is Measured or Detected

See KE Occurrence, Focal Seizure.

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Event: 389: Increased, Intracellular Calcium overload

Short Name: Increased, Intracellular Calcium overload

Key Event Component

Process	Object	Action
calcium ion transport	calcium ion	increased

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:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Please see KE Calcium influx, Decreasedin the AOP entitled *Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities.*

Additional text, specific for the AOP "Acetylcholinesterase Inhibition leading to Neurodegeneration":

Zebrafish have shown dysregulation in intracellular calcium ion levels following exposure to organophosphate compounds through similar mechanisms demonstrated in mammals (Faria et al. 2015).

Key Event Description

NMDAR agonist binding results in increased intracellular calcium, whereas NMDAR antagonist binding results in decreased intracellular calcium levels. For the relevant paragraphs below please see AOP entitled *Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities.*

Biological state: KE Calcium influx, Decreased

Biological compartments: KE Calcium influx, Decreased

General role in biology: KE Calcium influx, Decreased

The text specific for the AOP "ionotropic glutamatergic receptors and cognition" and "Acetylcholinesterase inhibition leading to neurodegeneration":

It is now well accepted that modest activation of NMDARs leading to modest increases in postsynaptic calcium are optimal for triggering LTD (Lledo et al. 1998; Bloodgood and Sabatin, 2007; Bloodgood et al. 2009), whereas much stronger activation of NMDARs leading to much larger increases in postsynaptic calcium are required to trigger LTP (Luscher and Malenka, 2012; Malenka 1994). Indeed, high-frequency stimulation causes a strong temporal summation of the excitatory postsynaptic potentials (EPSPs), and depolarization of the postsynaptic cell is sufficient to relieve the Mg2+ block of the NMDAR and allow a large amount of calcium to enter into the postsynaptic cells. Therefore, intra-cellular calcium is measured as a readout for evaluation NMDAR stimulation.

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?

Please see KE Calcium influx, Decreasedin the AOP entitled: *Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities.*

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Event: 55: Cell injury/death

Short Name: Cell injury/death

Key Event Component

Process	Object	Action
cell death		increased

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:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:278 - IKK complex inhibition leading to liver injury	KeyEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:265 - Uncoupling of oxidative phosphorylation leading to growth inhibition via increased cytosolic calcium	KeyEvent
Aop:264 - Uncoupling of oxidative phosphorylation leading to growth inhibition via ATP depletion associated cell death	KeyEvent
Aop:266 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased Na-K ATPase activity	KeyEvent
Aop:268 - Uncoupling of oxidative phosphorylation leading to growth inhibition via mitochondrial swelling	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to heart failure via increased myocardial oxidative stress	KeyEvent
Aop:490 - Co-activation of IP3R and RyR leads to economic burden through reduced IQ and non-cholinergic mechanisms	KeyEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
human and other cells in culture	human and other cells in culture	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Cell death is an universal event occurring in cells of any species (Fink and Cookson,2005).

Key Event Description

Two types of cell death can be distinguished by morphological features, although it is likely that these are two ends of a spectrum with possible intermediate forms. Apoptosis involves shrinkage, nuclear disassembly, and fragmentation of the cell into discrete bodies with intact plasma membranes. These are rapidly phagocytosed by neighbouring cells. An important feature of apoptosis is the requirement for adenosine triphosphate (ATP) to initiate the execution phase. In contrast, necrotic cell death is characterized by cell swelling and lysis. This is usually a consequence of profound loss of mitochondrial function and resultant ATP depletion, leading to loss of ion homeostasis, including volume regulation, and increased intracellular Ca2+. The latter activates a number of nonspecific hydrolases (i.e., proteases, nucleases, and phospholipases) as well as calcium dependent kinases. Activation of calpain I, the Ca2+-dependent cysteine protease cleaves the death-promoting Bcl-2 family members Bid and Bax which translocate to mitochondrial membranes, resulting in release of truncated apoptosis-inducing factor (tAIF), cytochrome c and endonuclease in the case of Bid and cytocrome c in the case of Bax. tAIF translocates to cell nuclei, and together with cyclophilin A and phosphorylated histone H2AX (γH2AX) is responsible for DNA cleavage, a feature of programmed necrosis. Activated calpain I has also been shown to cleave the plasma membrane Na+–Ca2+ exchanger, which leads to build-up of intracellular Ca2+, which is the source of additional increased intracellular Ca2+. Cytochrome c in cellular apoptosis is a component of the apoptosome.

DNA damage activates nuclear poly(ADP-ribose) polymerase-1(PARP-1), a DNA repair enzyme. PARP-1 forms poly(ADP-ribose) polymers, to repair DNA, but when DNA damage is extensive, PAR accumulates, exits cell nuclei and travels to mitochondrial membranes, where it, like calpain I, is involved in AIF release from mitochondria. A fundamental distinction between necrosis and apoptosis is the loss of plasma membrane integrity; this is

integral to the former but not the latter. As a consequence, lytic release of cellular constituents promotes a local inflammatory reaction, whereas the rapid removal of apoptotic bodies minimizes such a reaction. The distinction between the two modes of death is easily accomplished in vitro but not in vivo. Thus, although claims that certain drugs induce apoptosis have been made, these are relatively unconvincing. DNA fragmentation can occur in necrosis, leading to positive TUNEL staining (see explanation below). Conversely, when apoptosis is massive, it can exceed the capacity for rapid phagocytosis, resulting in the eventual appearance of secondary necrosis.

Two alternative pathways - either extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) - lead to apoptotic cell death. The initiation of cell death begins either at the plasma membrane with the binding of TNF or FasL to their cognate receptors or within the cell. The latter is due to the occurrence of intracellular stress in the form of biochemical events such as oxidative stress, redox changes, covalent binding, lipid peroxidation, and consequent functional effects on mitochondria, endoplasmic reticulum, microtubules, cytoskeleton, or DNA. The intrinsic mitochondrial pathway involves the initiator, caspase-9, which, when activated, forms an “apoptosome” in the cytosol, together with cytochrome c, which translocates from mitochondria, Apaf-1 and dATP. The apoptosome activates caspase-3, the central effector caspase, which in turn activates downstream factors that are responsible for the apoptotic death of a cell (Fujikawa, 2015). Intracellular stress either directly affects mitochondria or can lead to effects on other organelles, which then send signals to the mitochondria to recruit participation in the death process (Fujikawa, 2015; Malhi et al., 2010). Constitutively expressed nitric oxide synthase (nNOS) is a Ca2+-dependent cytosolic enzyme that forms nitric oxide (NO) from L-arginine, and NO reacts with the free radical such as superoxide (O2–) to form the very toxic free radical peroxynitrite (ONOO–). Free radicals such as ONOO–, O2 – and hydroxyl radical (OH–) damage cellular membranes and intracellular proteins, enzymes and DNA (Fujikawa, 2015; Malhi et al., 2010; Kaplowitz, 2002; Kroemer et al., 2009).

How it is Measured or Detected

Necrosis:

Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged. To detect the leakage of LDH into cell culture medium, a tetrazolium salt is used in this assay. In the first step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is converted to a colored formazan product using newly synthesized NADH in the presence of an electron acceptor. The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to enumerate the percentage of necrotic cells in a sample (Chan et al., 2013).

The MTT assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Other closely related tetrazolium dyes include XTT, MTS and the WSTs. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light (Berridgeet al.,2005).

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule used to stain necrotic cells. It is cell membrane impermeant so it stains only those cells where the cell membrane is destroyed. When PI is bound to nucleic acids, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm (Moore et al.,1998)

Alamar Blue (resazurin) is a fluorescent dye. The oxidized blue non fluorescent Alamar blue is reduced to a pink fluorescent dye in the medium by cell activity (O'Brien et al., 2000) (12).

Neutral red uptake, which is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in lysosomes (Repetto et al., 2008) (13). Moreover, quantification of ATP, signaling the presence of metabolically active cells, can be performed (CellTiter-Glo; Promega).

ATP assay: Quantification of ATP, signaling the presence of metabolically active cells (CellTiter-Glo; Promega).

Apoptosis:

TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase or TdT, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage.

Caspase activity assays measured by fluorescence. During apoptosis, mainly caspase-3 and -7 cleave PARP to yield an 85 kDa and a 25 kDa fragment. PARP cleavage is considered to be one of the classical characteristics of apoptosis. Antibodies to the 85 kDa fragment of cleaved PARP or to caspase-3 both serve as markers for apoptotic cells that can be monitored using immunofluorescence (Li, Peng et al., 2004).

Hoechst 33342 staining: Hoechst dyes are cell-permeable and bind to DNA in live or fixed cells. Therefore, these stains are often called supravital, which means that cells survive a treatment with these compounds. The stained, condensed or fragmented DNA is a marker of apoptosis (Loo, 2002; Kubbies and Rabinovitch, 1983).

Acridine Orange/Ethidium Bromide staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis.

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List of Adverse Outcomes in this AOP

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

Stressors

Name
Sars-CoV-2
Chemical
SARS-CoV
Virus

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

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

Term	Scientific Term	Evidence	Links
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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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 11: AchE Inhibition leads to ACh Synaptic Accumulation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase inhibition leading to acute mortality	adjacent	High	Moderate
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	High	Moderate
Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	adjacent		
Organo-Phosphate Chemicals induced inhibition of AChE leading to impaired cognitive function	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Metapenaeus monoceros	Metapenaeus monoceros	High	NCBI
Philosamia ricini	Samia ricini	High	NCBI
Rana cyanophlyetis	Euphlyctis cyanophlyctis	Moderate	NCBI
Tilapia mossambica	Oreochromis mossambicus	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
zebrafish	Danio rerio	Moderate	NCBI
Japanese quail	Coturnix japonica	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	High

Cholinergic transmissions mediated by acetylcholinesterase occur in a wide variety of species, both vertebrates and invertebrates, and cholinergic transmissions occur at all stages in life.

Taxonomic Applicability

- The literature includes many studies linking increases in acetylcholine in brain tissues after exposure to an OP or carbamate pesticide with increased AChE inhibition in various taxa. Examples include studies with crustacea (Reddy et al., 1990); tadpoles (Nayeemunnisa and Yasmeen, 1986); fish (Rao and Rao 1984; Verma et al., 1981); birds (Kobayashi et al., 1983); and rodents (Kobayashi et al., 1988).

Key Event Relationship Description

- AChE is an enzyme responsible for controlling the level of acetylcholine available at cholinergic synapses by degrading this neurotransmitter via hydrolysis to acetic acid and choline (Wilson 2010). Inhibition of AChE prevents degradation of acetylcholine which leads to accumulation of acetylcholine in synapses associated with muscarinic and nicotinic receptors (Soreq and Seidman, 2001; Lushington 2006).
- See KEGG Reaction R01026

Evidence Supporting this KER

Biological Plausibility

- Acetylcholine is a critical neurotransmitter localized to neuronal synapses. Biological plausibility to support the relationship between AChE inhibition and accumulation of acetylcholine is rooted in evidence demonstrating that AChE catalyzes degradation of acetylcholine into choline and acetate. Therefore, inhibition of the AChE leads to acetylcholine accumulation.

Empirical Evidence

- In a study where female ICR mice were exposed to either the fenobucarb or propoxur, authors reported a significant increase in acetylcholine in brain tissue 10 minutes after injection, with a concurrent significant increase in AChE inhibition (Kobayashi et al., 1985).
- An acute (48h) sublethal exposure to methyl parathion found that AChE levels in brain tissue in fish (Oreochromis mossambicus) were significantly inhibited at all measured durations ranging from 12-48 hrs with inhibition increasing from 36-62% as compared to controls over the time span (Rao and Rao, 1984). The researchers found a significant increase in acetylcholine at all time courses measured (12-48hr) with acetylcholine levels increasing from 33-83% as compared to controls over the same time span (Rao and Rao, 1984).
- A study of quail (Coturnix japonica) exposed to lethal concentrations of two OP pesticides (i.e., DDVP or fenitrothion), found significant increases in total and free acetylcholine, and significant inhibition of AChE as compared to controls (Kobayashi et al., 1983).
- Measurements (in vitro) of AChE inhibition, acetylcholine and electrophysiological responses on the pedal ganglion of the gastropod Aplysia californica, were found to be dose-dependent, with increase in dose resulting in increased AChE inhibition, increased levels of acetylcholine, and a decrease in the electrophysiological response (Oyama et al., 1989).
- Wister rats injected with a sublethal concentration of dichlorvos found a significant decrease in AChE activity, increased acetylcholine concentrations, and enhanced contractile responses in jejunum muscle (Kobayashi et al., 1994).
- At sublethal concentrations (56% of the LD50), researchers found a statistically significant (18%) increase in the amount of acetylcholine in brain tissue of Charles River rats exposed to disulfoton for 3 days, with measured AChE inhibition of 68% as compared to controls (Stavinoha et al., 1969).
- An acute sublethal exposure of chlorpyrifos to Sprague-Dawley rats found significant dose and time related effects including increased inhibition of AChE, increased levels of acetylcholine, and significant impacts to motor activity (nocturnal rearing response) (Karanth et al., 2006).
- Tadpoles (20 d) were exposed to single sublethal concentration of the methyl parathion for 24 h. Analysis of brain tissue found a significant inhibition in AChE activity and a concurrent increase in acetylcholine levels, as compared to controls (Nayeemunnisa and Yasmeen 1986).
- Study of fourth instar *Ailanthus* silkworm exposed to malathion for 5 days found increased mortality, decreased AChE, and increases in acetylcholine as compared to controls (Pant and Katiyar 1983).

- Faria et al (2015) exposed zebrafish (*Danio rerio*) larvae to different concentrations of chlorpyrifos oxon (CPO). A strong inhibitory effect on AChE activity was found as early as 1h after exposure with a 50% inhibitory concentration (IC50) of 64 nm CPO. The authors showed that the zebrafish model mimicked most of the effects seen in humans, including AChE inhibition, calcium dysregulation, ad inflammatory and immune responses.

Uncertainties and Inconsistencies

- No known qualitative inconsistencies or uncertainties associated with this relationship.

Quantitative Understanding of the Linkage

The general kinetic equation is:

$$\mathrm{AChE} + \mathrm{AX} \underset{}{\overset{K_1}{\rightleftharpoons}} \mathrm{AChEAX} \xrightarrow{K_2} \mathrm{AChEA} + \mathrm{X} \xrightarrow{K_3} \mathrm{AChE} + \mathrm{A}$$

- Where AX is the substrate, either acetylcholine or an inhibitor of AChE (e.g., OP or carbamate);
- AChE-AX is the enzyme-substrate complex;
- AChE-A is the acylated, carbamylated or phosphorylated enzyme;
- X is the leaving group (e.g., choline);
- AChE is the free enzyme; and
- A is acetic acid, phosphate (P(=O)(=O)(R2)or methylamine.
- In a normally functioning enzyme system k1 is the rate-limiting step for hydrolysis of acetylcholine, but k3 is the rate limiting step when AChE is inhibited by carbamates or OPs (Wilson 2010).
- Some rate constants for OPs and carbamates have been published for use in PBPK models (Knaak et al., 2004, 2008)

Table 1: Summary of available quantitative data describing responses of ACh to AChE inhibition. Data are grouped by species.

AChE Inhibitor	CAS RN	Inhibitor Dosage	Species / Model	Brief Summary	Refer
Donezepil	120014-06-4	0.625, 1.25, 2.5 (mg/kg)	Male Wistar rats (210-290 g | 7 weeks)	Timecourse data on both extracellular hippocampal ACh concentration and AChE activity given varying concentrations of inhibitor. Brain concentrations of drugs over time are also provided.	Kosasa 19
Tacrine	321-64-2	1.25, 2.5, 5, 10 (mg/kg)			
ENA-713 (Rivastigmine)	129101-54-8	0.625, 1.25, 2.5 (mg/kg)			
Dichlorvos (DDVP)	62-73-7	5 (mg/kg)	Male Wistar rats (180-230 g)	AChE activity (µmol AthCh hydrolyzed/g tissue) and ACh content (nmol ACh/g tissue) in jejunum either 10 minutes after single injection or 1 day after 10 injections.	Kobayas 19
Propoxur	114-26-1	10 (mg/kg)			
			Wistar rats (fetal days 17-	*In Vitro* AChE activity (% control) and ACh	

AChE Inhibitor	CAS RN	Inhibitor Dosage	Species / Model	Brief Summary	Refer
Paraoxon (PO)	311-45-5	0.1, 1, 10, 20, 30 (µM)	Primary hippocampal neurons	*In Vitro* AChE activity (% control) and ACh concentration (pmol/well) at 1 hour and 14 days post exposure	Del Pino 20
Tacrine	321-64-2	1.25, 2.5, 5 (mg/kg)	Male Wistar rats (210-290 g | 6 weeks)	Timecourse data on both extracellular hippocampal ACh concentration and AChE activity given varying concentrations of inhibitor. Note: Several sections of text are verbatim from Kosasa et al., 1999.	Kim 2
Parathion (PS)	56-38-2	adult: 1.8, 3.4, 6, 9, 18, 27 (mg/kg) aged: 1.8, 3.4, 6, 9 (mg/kg)	Male Sprague-Dawley rats (adult: 3 months) (aged: 18 months)	Diaphragm and striatum AChE activity (% control). Striatal dialysates of ACh (fmol/60 µL fraction) on day 3 and 7 post-exposure	Karanth 20
Chlorpyrifos (CPF)	2921-88-2	84, 156, 279 (mg/kg)	Male Sprague-Dawley rats (325-350 g | 3 months)	Diaphragm and striatum cholinesterase activity (% control). ACh concentration (fmol/60 µL fraction) through *In Vivo* microdialysis at 1, 4, and 7 days post-exposure	Karanth 20
Paraoxon	311-45-5	0.03, 0.1, 1, 10 (µM)	Male Sprague-Dawley rats (275-299 g | 2-3 months)	Timecourse data on changes in striatal AChE activity (% control) and ACh concentration (fmole/fraction (60 µL)) over 4 hours post exposure.	Ray et a
Propoxur	114-26-1	10 (mg/kg)	Female ICR mice (30-40 g | 8-10 weeks)	AChE activity (µmol acetylthiocholine hydrolyzed /min/g wet tissue) and ACh content (nmol/g wet tissue) both measured at 0, 10, 60, 180 minutes after injection (and 360 minutes for AChE activity)	Kobayas 19
BPMC	3766-81-2	10 (mg/kg)	Female ICR mice (30-40 g | 8-10 weeks)	Timecourse data on AChE activity (µmole acetylthiocholine hydrolyzed / min / g tissue or ml blood) and ACh content (nmol/g tissue) of forebrain homogenate, taken at 0, 10 and 60 minutes.	Kobayas 19
Propoxur	114-26-1	2 (mg/kg)			
DE-71	32534-81-9	31.0, 68.7, 227.6 (µg/L)	Zebrafish larvae	Changes in AChE activity (nmol / min / mg protein) and ACh concentration (nmol / mg protein) measured at 120 hours post-fertilization	Chen et
Dichlorvos (DDVP)	62-73-7	3 (mg/kg)	Male Japanese quail (100 g | 8-14 weeks)	AChE activity (µmol ACh hydrolyzed/g) and ACh content (nmol ACh/g wet tissue) measured 10 and 60 minutes post exposure for DDVP and Fenitrothion, respectively.	Kobayas 19
Fenitrothion	122-14-5	300 (mg/kg)			
Methyl Parathion	298-00-0	0.09 (ppm)	*Tilapia mossambica*	Timecourse data on AChE activity (µmol ACh hydrolysed/mg protein/h) and ACh content (µmole/g wt. tissue) in muscle, gill, liver, and brain tissue at 12, 24, 36, and 48 hr timepoints	Rao an 19
Methyl Parathion	298-00-0	2.5 (ppm)	*Rana cyanophilicitus* Frog tadpole (1.5-2 g | 20 days)	AChE activity (µmol ACh hydrolyzed /min) and ACh content (µmol/g) measured after 24 hours post exposure	Yasme Yasmee
Malathion	121-75-5	60 µg each/g insect weight/day	*Philosamia Ricini larvae*	AChE activity and ACh concentration changes measured daily for 5 days.	Pant and 19

Response-response relationship

Striatal AChE activity and extracellular ACh levels were measured in rats intracerebrally perfused with paraoxon (0, 0.03, 0.1, 1, 10 or 100 µM, 1.5 µl/min for 45 min). Acetylcholine was below the limit of detection at the low dose of paraoxon (0.1 uM), but was transiently elevated (0.5–1.5 hr) with 10 µM paraoxon. Concentration-dependent AchE inhibition was noted but reached a plateau of about 70% at 1 µM and higher concentrations (Ray, 2009).

Time-scale

The relationship between AChE inhibition and ACh accumulation at the synapse can be observed within 30 minutes after application of an AChE inhibitor (Ray, 2009). Other experiments have shown significant differences in ACh after AChE inhibition as soon as an hour after application of a chemical stressor (Kim et al., 2003, Faria et al., 2015).

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
enzyme	butylcholinesterase	Butylcholinesterase can affect the substrate interaction and should be accounted for	Wilson (2001)

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Relationship: 1857: ACh Synaptic Accumulation leads to Activation, Muscarinic Acetylcholine Receptors

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	Moderate	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
zebrafish	Danio rerio	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Muscarinic receptors are found in a wide variety of species, both vertebrates and invertebrates, and cholinergic transmissions occur at all stages in (Burke et al., 2017, Garcia et al., 2016, Miller and Yeh, 2016).

Key Event Relationship Description

Acetylcholine (ACh) is a neurotransmitter within the central nervous system and peripheral nervous system that activates both muscarinic and nicotinic receptors (Haga, 2013). Muscarinic receptors are metabotropic and act using slower transmission signaling compared to the more direct ionotropic receptors (Miller and Yeh, 2016).

Evidence Supporting this KER

Binding of Ach to muscarinic receptors has been well documented to activate the receptor (Miller and Yeh, 2016). Using radiolabeled ACh ([3H]-ACh), experimenters have determined the binding kinetics between ACh and muscarinic receptors (Kellar et al., 1985, Uchida et al., 1978). Additionally, a computational model was recently developed modeling a CA1 pyramidal neuron's response to activation of M1 receptors in the presence of ACh (Mergenthal et al., 2020)

Biological Plausibility

It is well known that muscarinic receptors bind ACh. Muscarinic receptors are found in the target organs of parasympathetic neurons and in various parts of the central nervous system (Haga, 2013). Muscarinic receptors expressed in the brain are the M1, M2, and M4 subtypes more than the M3 or M5 subtypes (Lebois et al., 2018).

Empirical Evidence

- Symptoms from increasing ACh levels are partially reduced when pretreated with muscarinic antagonists like atropine (Faria et al., 2015, King and Aaron, 2015).
- Rats and rabbits pretreated with a combination of Neostigmine or Physostigmine (reversible AChE inhibitor), Mecamylamine (nicotinic receptor antagonist), or Atropine (mAChR antagonist) and later exposed to Soman, a strong irreversible AChE inhibitor, showed a significantly increased survival rate and overall reduced brain ACh levels compared to the control group (Harris et al., 1980).

Uncertainties and Inconsistencies

There are no known uncertainties or inconsistencies with this relationship.

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Relationship: 1889: Activation, Muscarinic Acetylcholine Receptors leads to Occurrence, Focal Seizure

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
guinea pig	Cavia porcellus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

M1 activation leading to focal seizure activity appears in many different species, both genders, and at various life stages. Specific experiments are listed under the empirical evidence above.

Key Event Relationship Description

Muscarinic receptors are metabotropic, affecting a target enzyme which typically sends secondary messenger signals (Kandel et al., 2013). Pharmacological evidence indicates the mAChR M1 subtype modulates the M current in sympathetic ganglion neurons. In mice, M1 agonists suppress the M current and results in membrane depolarization that leads to focal seizures (Hamilton et al., 1997). Seizures occurring through the M1 muscarinic receptor have been observed to start at 5-15 minutes after exposure in rats and guinea pigs (Miller, 2015, Sparenborg et al., 1992).

Evidence Supporting this KER

Biological Plausibility

M1 Muscarinic receptors are modulators of M-current potassium channel activity (Marrion, 1997). Blocking the M-current through the M1 receptor contributes to cell depolarization, which then leads to the start of epileptiform activity (Greget et al., 2016). The use of muscarinic agonists is well established and often used in animal models of epilepsy and include compounds such as pilocarpine and carbachol (Curia et al., 2008, Turski et al., 1983). It has been suggested that seizures initiated through M1 receptor activation occur when the ratio between glutamatergic and GABAergic activity reaches a threshold (Miller, 2015).

Empirical Evidence

- Investigations into receptors involved in cholinergic seizures have found that pre-treatment with the selective M1 antagonist pirenzepine abolished seizures in 91% of the rats tested. The drugs mecamylamine, a nicotinic antagonist, and methoctramine, a M1 receptor antagonist, did not significantly affect seizure activity (Cruickshank et al., 1994).
- Muscarinic antagonists were effective in lessening the seizure activity in guinea pig hippocampal slices (Harrison et al., 2004).
- M1- deficient mice neurons lacked the modulation of M-current caused by muscarinic agonists that is shown in wild-type mice. The mice lacking M1 receptors are also resistant to pilocarpine-induced seizures (Hamilton et al., 1997).
- In a rat study, pretreatment with atropine, a mAChR antagonist, was able to prevent cholinergic symptoms such as convulsions and acute mortality following an injection of physostigmine, a reversible AChE inhibitor (Davis and Hatoum, 1980).

Uncertainties and Inconsistencies

Experiments blocking the M2 subtype have not led to a decrease in seizures from acetylcholinesterase inhibitors, only the antagonist for M1 subtype decreased seizure activity (Cruickshank et al., 1994). This demonstrates that the M1 subtype is vital for muscarinic receptor caused seizures. Many studies have noted that delaying administration of M1 antagonist, even for just a short amount of time after exposure, does not halt status epilepticus development (Miller, 2015). This indicates that M1 receptors are not responsible for maintaining seizure activity, they are only responsible for the initial phase (Hamilton et al., 1997). The secondary generalization of the focal seizure is continued by some other mechanism.

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Relationship: 1890: Occurrence, Focal Seizure leads to Increased, glutamate

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	Low	NCBI
guinea pig	Cavia porcellus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

This relationship has been demonstrated in rats, and human toxicity through this pathway has also been indicated (King and Aaron, 2015).

Key Event Relationship Description

The initial focal seizure starts by increasing the firing rate of neurons in a specific area. This is characterized by changes in membrane potential (Turski et al., 1986). Cholinergic nerve agents cause an increase in spontaneous excitatory postsynaptic currents (sEPSC) leading to increased release of glutamate and activation of N-methyl-D-aspartate receptors (NMDARs) (Lallement et al., 1991, Miller, 2015). This response happens quickly after the initial focal seizure and is then sustained for a longer period of time (McDonough and Shih, 1997).

Evidence Supporting this KER

Seizure activity has been shown to cause glutamate release (Lallement et al., 1991). Glutamate is the main excitatory transmitter in the brain and spinal cord, where it activates both ionotropic and metabotropic receptors (Kandel et al., 2013).

Biological Plausibility

Glutamate (Glu) release into the synaptic cleft is primarily caused by excitatory glutamatergic neurons, however there is evidence showing astrocytes releasing glutamate through a calcium-dependent process (Nedergaard et al., 2002). A mechanism explaining how astrocytes release glutamate is not well defined, but it could be released through exocytosis (Nedergaard et al., 2002). When focal seizures start, the firing of glutamatergic neurons releases glutamate (Lallement et al., 1991). While the change in spiking activity of individual neurons at seizure onset appears to be heterogenous, there is an apparent increase in neuronal firing rate in some populations of neurons (Truccolo et al., 2011).

Empirical Evidence

- Exposure to nerve agents that induce seizures also increases free glutamate levels. Rats that did not experience seizures also did not have increased free glutamate levels (Lallement et al., 1991)
- Experiments on rats have indicated delayed changes in free glutamate levels after seizure onset, indicating that the activity is in response to seizure activity, not initializing it (McDonough and Shih, 1997).

Uncertainties and Inconsistencies

There is not yet an explanation for the mechanisms behind glutamate release in response to seizure activity. Animals that developed seizure activity in response to sarin (aka GB) versus VX intoxication showed increasing extracellular glutamate and no changes in extracellular glutamate, respectively (O'Donnell et al., 2011).

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Relationship: 1859: Increased, glutamate leads to Overactivation, NMDARs

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	Moderate	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

This relationship has been demonstrated in rats, and human toxicity through this pathway has also been indicated (King and Aaron, 2015).

Key Event Relationship Description

Glutamate is the main excitatory neurotransmitter in the brain and spinal cord, where it activates both ionotropic and metabotropic receptors (Kandel et al., 2013). N-methyl-D-aspartate (NMDA) receptors are one class of ionotropic glutamate receptors found in the brain. They are unique in that they require multiple ligands, both glutamate and glycine, to first bind before they can open. Under normal conditions, the extracellular concentration of glycine is high enough to allow effective opening of NMDA receptors by glutamate (Kandel et al., 2013). NMDA receptors are also voltage-gated by a magnesium block and requires depolarization of the neuron to which the NMDA receptors are bound before ions can flow through the receptor channel (Kandel et al., 2013). A variety of pathological conditions involve the overactivation of glutamate receptors and result in some form of injury (Lipton and Rosenberg, 1994). For example, elevated extracellular glutamate levels have been shown to occur during periods of seizure activity (Lallement et al., 1991). Excess extracellular glutamate is known to be toxic to neurons and can result in cell death due to calcium dysregulation mediated through NMDA receptor activation (Michaels and Rothman, 1990).

Evidence Supporting this KER

Biological Plausibility

Glutamate release into the synaptic cleft is primarily caused by excitatory glutamatergic neurons, however there is evidence showing astrocytes releasing glutamate through a calcium-dependent process. A mechanism explaining how astrocytes release glutamate is not well defined, but it could be released through exocytosis (Nedergaard et al. 2002). Excessive extracellular glutamate overactivates NMDARs and propagates the excitotoxicity caused by some nerve agents (McDonough and Shih, 1997).

Empirical Evidence

- Pretreatment with NMDA receptor antagonist MK-801 delayed cell injury and death induced by glutamate toxicity (Michaels and Rothman, 1990).

A rat study by Smolders et al. (1997) demonstrated that seizures initiated by pilocarpine were further mediated through NMDA receptors and that these seizures were terminated upon administration of MK-801, an NMDA receptor antagonist.

Uncertainties and Inconsistencies

There are no known uncertainties or inconsistencies with this relationship.

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Relationship: 2781: Overactivation, NMDARs leads to Status epilepticus

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
guinea pig	Cavia porcellus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The effect of overactivation of NMDA receptors leading to seizure activity has been shown in vertebrate species. Notably, *in vivo* evidence is provided above in empirical evidence for both rats and guinea pigs (Borris et al., 2000, Braitman and Sparenborg, 1989, Mazarati and Wasterlain, 1999, Sparenborg et al., 1992)

Key Event Relationship Description

N-methyl-D-aspartate (NMDA) receptors are ligand and voltage-gated receptors. They require both a post-synaptic depolarization to remove the Mg^{2+} block and binding of glutamate for receptor activation (Kandel et al., 2013). High frequency stimulation that occurs during seizure activity aides in removal of the Mg^{2+} block through depolarization of the affected neuron. The conditions for NMDA receptor activation are ideal as there is prolonged firing and overall increased depolarization during seizure activity (Kapur, 2018). Sustainment of seizure activity for greater than 5 minutes develops into status epilepticus (McDonough and Shih, 1997).

Evidence Supporting this KER

Biological Plausibility

NMDA receptor activation through intraperitoneal administration of NMDA has been shown to cause seizure activity in developing and young adult rats with a marked decrease in effect with age (Mares and Velísek, 1992). NMDA receptors appear to play a role in the upregulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and downregulation of gamma-aminobutyric acid A ($GABA_A$) receptors through calcium dependent mechanisms during status epilepticus, which ultimately aids in the self-sustainment of seizure activity (Joshi et al., 2017, Kapur, 2018). Furthermore, seizure termination can be seen to occur with application of NMDA receptor antagonists, as evidenced below.

Empirical Evidence

- Status epilepticus induced by exposure to soman in guinea pigs either reduced or arrested after treatment with MK-801, an NMDA receptor antagonist, or was prevented entirely by pretreatment with MK-801 (Sparenborg et al., 1992). An earlier experiment under the same conditions showed similar results (Braitman and Sparenborg, 1989).
- Status epilepticus was induced through electrical stimulation in 13–15-week-old male Wistar rats and was successfully terminated using MK-801 (Mazarati and

Wasterlain, 1999).
- Adult male Sprague-Dawley rats had status epilepticus induced through electrical stimulation which was shown to be refractory to GABAergic drugs, but was still successfully terminated using ketamine, an NMDA receptor antagonist (Borris et al., 2000).

Uncertainties and Inconsistencies

Rats exposed to diisopropylfluorophosphate (DFP) manifested status epilepticus and did not respond to treatment with MK-801 (Deshpande et al., 2010). This is in contrast to the effects seen in the above experiments involving soman or electrical stimulation (Mazarati and Wasterlain, 1999, Sparenborg et al., 1992), suggesting that DFP may include mechanisms that are involved in the initiation and/or maintenance of seizure activity.

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Relationship: 2782: Status epilepticus leads to Increased, glutamate

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
guinea pig	Cavia porcellus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

See KER 1890: Occurrence, Focal Seizure leading to Increased, glutamate.

Key Event Relationship Description

Sustained seizure activity that lasts longer than 5 minutes, or repetitive seizures without regaining consciousness constitute status epilepticus (Lowenstein and Alldredge, 1998). Release of glutamate through this sustained seizure activity follows that of KER 1890: Occurrence, Focal Seizure leading to Increased, glutamate.

For AChE inhibition-induced status epilepticus, there are in total three points that differentiate the key events of focal seizure onset (KE 1623) and status epilepticus in the AChE inhibition-induced model of seizure activity: (i) Focal seizures are localized seizures that have not spread/undergone secondary generalization (Kandel et al., 2013). (ii) Status epilepticus has specifically defined requirements that must be met for a subject to be considered to be in status epilepticus, those being that the seizure(s) must have lasted for at least 5 minutes or there are repetitive seizures occurring without the subject regaining function and consciousness (Lowenstein and

Alldredge, 1998). The transition between focal seizure activity and generalized status epilepticus occurs somewhere between 5 and 40 minutes after seizure onset (McDonough and Shih, 1997) (iii) The treatment options available for attenuating seizure activity induced by AChE inhibition are best when the seizures initially begin as a focal seizure and reduced when the subject has been in the state of status epilepticus for a prolonged period of time. Specifically, in the early phases of the pathology after exposure to the AChE inhibitor, a cholinergic phase is present, and effective treatment options include both regular anti-seizure treatment and anticholinergic therapy to prevent the seizures from continuing, whereas in the later phase of the pathology, where the seizure activity is now glutamatergically driven, anticholinergic therapy is no longer effective, and the seizure activity can only be effectively treated with the usual therapies (McDonough and Shih, 1997).

Evidence Supporting this KER

Increases in glutamate release have been shown to occur after the onset of seizure activity (Lallement et al., 1992). See Table 1 in KER 1890: Occurrence, Focal Seizure leading to Increased, glutamate for experiments that measure both seizure activity via electroencephalogram (EEG), and extracellular glutamate during seizure activity.

Biological Plausibility

See KER 1890: Occurrence, Focal Seizure leading to Increased, glutamate.

Empirical Evidence

See KER 1890: Occurrence, Focal Seizure leading to Increased, glutamate.

Uncertainties and Inconsistencies

See the KER Occurrence, Focal Seizure leading to Increased, glutamate. Additionally, for organophosphate-induced status epilepticus, it is uncertain when the shift from cholinergic driven processes change to glutamatergic processes. There is a transitional phase where modulation gradually is transferred from cholinergic to noncholinergic mechanisms (McDonough and Shih, 1997).

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Relationship: 361: Overactivation, NMDARs leads to Increased, Intracellular Calcium overload

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	Moderate	
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	High	Moderate
Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	adjacent	Not Specified	Not Specified
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	Low	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

NMDARs have been shown to regulate calcium ion flow in a variety of species including zebrafish and rats (Horzmann and Freeman, 2016, el Nasr et al., 1990).

Key Event Relationship Description

The NMDA receptor is distinct from the other glutamate receptors in two ways: first, it is both ligand-gated and voltage-dependent; second, it requires co-activation by two ligands: glutamate and either glycine or D-serine. Following membrane depolarization, the co-agonists, L-glutamate and glycine must bind to their respective sites on the receptor to open the channel. On activation, the NMDA receptor allows the influx of extracellular calcium ions into the postsynaptic neuron and neurotransmission occurs (reviewed in Higley and Sabatini, 2012). Calcium flux through NMDA receptors is also thought to be critical in synaptic plasticity, a cellular mechanism for learning and memory. Indeed, NMDA receptor–dependent synaptic potentiation (LTP) and depression (LTD) are two forms of activity-dependent long-term changes in synaptic efficacy that are believed to represent cellular correlates of learning and memory processes. The best characterized form of NMDA receptor-dependent LTP and LTD occurs between CA3 and CA1 pyramidal neurons of the hippocampus (Luscher and Malenka, 2012). It is now well established that modest activation of NMDARs leads to modest increases in postsynaptic calcium, triggering LTD, whereas much stronger activation of NMDARs leading to much larger increases in postsynaptic calcium are required to trigger LTP (Luscher and Malenka, 2012). The high-frequency stimulation causes a strong temporal summation of the excitatory postsynaptic potentials, and depolarization of the postsynaptic cell is sufficient to relieve the Mg2+ block of the NMDAR and allow a large amount of calcium to enter into the post-synaptic cells.

Evidence Supporting this KER

Biological Plausibility

There is structural and functional mechanistic understanding supporting this relationship between NMDAR overactivation and increased intracellular calcium.

The relationship between the upstream and downstream key event is plausible as the expression of the functional NMDA receptors is commonly carried out or assessed by Ca2+ imaging method. Calcium imaging techniques have been extensively utilized in the literature to investigate the potential interactions between NMDA-evoked Ca2+ influx and NMDA receptor activation. Approximately 15% of the current through NMDA receptors is mediated by Ca2+ under physiological conditions (Higley and Sabatini, 2012).

It has been shown that less than five and, occasionally, only a single NMDA receptor opens under physiological conditions, causing a total Ca2+ influx of about 6000 ions into a spine head reaching a concentration of ~10 µm (Higley and Sabatini, 2012). However, the majority of the ions are rapidly eliminated by binding to Ca2+ proteins, reaching ~1 µM of free Ca2+ concentration (Higley and Sabatini, 2012).

It has been shown that in rat primary forebrain cultures the intracellular Ca2+ increases after activation of the NMDA receptor through administration of NMDA but this increase in Ca2+ is blocked when the cells are cultured under Ca2+ free conditions, demonstrating that the NMDA-evoked increase in intracellular Ca2+ derives from extracellular and not intracellular sources (Liu et al., 2013).

Indirect mechanism of domoic acid (DA) induced overactivation of NMDARs that result in Ca2+ overload: depolarization of the pre-synaptic cell activates the release of endogenous Ca2+ which mobilizes vesicles containing GLU to the membrane surface. Glutamate (GLU) is then released into the synaptic cleft by exocytosis where it is able to interact with cell surface receptors. Exogenous DA can interact within the synaptic cleft with each of the three ionotropic receptor subtypes including the kainate, AMPA, and NMDA receptors on cell membranes. Activation of the kainate and AMPA receptors results in release of Ca2+ via coupled ion channels, into the post-synaptic cell. DA is also able to bind to NMDA receptors that are linked to both Ca2+ and NA/K+ ion channels and results in a cellular influx of both Na+ and Ca2+. Unlike GLU, DA induces prolonged receptor activation causing a constant influx of cations into the cell and the appropriate chemical cues for desensitization are blocked. The excess intracellular Ca2+ causes disruption of cellular function, cell swelling and ultimately cell death (Lefebvre and Robertson,2010).

Glufosinate (GLF) is the methylphosphinate analog of glutamate that directly can activate NMDARs (Lantz et al., 2014, Matsumura et al., 2001, Faro et al., 2013) (as described in KE: *NMDARs, Binding of agonist*). It is well established in the existing literature that activation of NMDARs leads to the intra-cellular Ca2+ overload and based on this assumption it can be suggested that an exposure to GLF leads to increased intra-cellular calcium levels.

Empirical Evidence

Include consideration of temporal concordance here

Domoic acid (DomA)

- Treatment of mouse cerebellar granule neurons (CGNs) with 1 or 10 µM DomA causes increase of intracellular Ca2+ by approximately 5 or 8 fold compared to controls, respectively (Giordano et al., 2006). Interestingly, when the cells are exposed simultaneously to DomA and the NMDA receptor antagonist MK-801, the Ca2+ levels measured are close to control levels, indicating that the Ca2+ elevation evoked by DomA involves activation of NMDA receptors (Giordano et al., 2006).
- The same research group has performed a time course study by applying a high and a low DomA concentration and using CGNs from Gclm (+/+) and Gclm (–/–) mice lacking glutathione (Giordano et al., 2007). The low DomA dose (0.1µM) causes a small and delayed increase in intracellular Ca2+ concentration with a full recovery by 20 min. When the experiment is performed in the absence of extracellular calcium, this increase of intracellular Ca2+ levels in the presence of DomA is abolished, indicating that this change in homeostasis of Ca2+ is due to ion entry from outside the cell. However, this recording of intracellular Ca2+ is antagonised only by NBQX (AMPA receptor antagonist), but not by MK-801 (NMDA receptor antagonist). On the other hand, the higher DomA concentration (10µM) causes a rapid and robust increase in intracellular Ca2+, which lasts even after 25 min. This effect is antagonized by both NBQX and MK-801, suggesting that not only AMPA but also NMDA receptors are involved in Ca2+ elevation evoked by DomA at high doses (Giordano et al., 2007).
- In an earlier study, the time course and concentration dependence of the increase in intracellular Ca2+ stimulated by DomA has been examined in 10-13 day-in-culture CGNs (Berman et al., 2002). DomA produces a rapid and concentration-dependent increase in intracellular Ca2+, showing the maximal increase at 10 µM DomA (Berman et al., 2002). At this concentration, fluo-3 fluorescence that is used to measure Ca2+ elevates rapidly during the first 40 s of exposure, increases more slowly before peaking at 3.5 min, after which the signal diminishes steadily over the 30 min course of the experiment to 55% of peak values. The EC50 for DomA-induced increase in intracellular Ca2+ is 0.61 µM. In the same study, the NMDA receptor antagonist MK-801 significantly reduced both peak and final plateau of intracellular Ca2+ by 30 and 70%, respectively (Berman et al., 2002).
- These three studies (Giordano et al., 2006; 2007; Berman et al., 2002) do not provide a simultaneous measurement of NMDA receptor activation by DomA and intracellular Ca2+ levels. However, they do provide indirect evidence of NMDA receptor activation involvement in increased intracellular Ca2+ concentrations induced by DomA as they have used known antagonists of the NMDA receptors that reverses the situation in both KEs (blocking upstream KE will block downstream KE).

- In an in vivo study it was indirectly shown that the microinjection to adult male Sprague Dawley rats of 10 µM DomA increased intracellular Ca2+ levels. A significant upregulation of phosphorylated calcium-calmodulin-dependent kinase II (CaMKII) and phosphorylated cAMP response element binding protein (CREB) levels was recorded, possibly due to increased intracellular Ca2+ levels induced by DomA (Qiu and Currás-Collazo, 2006).

In CGNs, the co-treatment with 10 µM DomA and the kainate/AMPA receptor antagonist NBQX maintains Ca2+ levels near to control levels, suggesting that the Ca2+ elevation evoked by DomA is mediated by the activation of both AMPA/kainate and of NMDA receptors (Giordano et al., 2006).

The voltage-sensitive Ca2+ channel (VSCC) blocker nifedipine (5 µM) and NBQX (10 µM), a competitive AMPA/kainate receptor antagonist reduces the peak and final intracellular Ca2+ concentration in CGNs (Berman et al., 2002), strengthening the view that the increase of Ca2+ influx is not only mediated by NMDA receptors but also by AMPA/kainate receptors and VSCCs.

Table 1: Summary of available data describing responses of intracellular calcium to NMDA receptor activation. DA, DomA = Domoic Acid. Glu = Glutamate. NMDA = N-methyl-D-aspartate. The following are NMDA receptor (NMDAR) antagonists: D-AP5 = D-2-amino-5-phosphonopentanoate. MK-801 = Dizocilpine.

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Overactivation of NMDAR (KE up) (measurements, quantitative if available)	Increased intracelllular Ca 2+ levels (KE down) (measurements, quantitative if available)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Mouse cerebellar granule neurons (CGNs) from Gclm (+/+) and Gclm (–/–) mice	0.01 to 10 µM		Time course (15 to 120 min)		5 and 8 fold increase of [Ca2+]i compared to controls.	Giordano et al., 2006				The cells were exposed simultaneously to DA and the NMDA receptor antagonist MK-801 and the Ca2+ levels were found to be close to control levels, indicating that the Ca2+ elevation evoked by DA involves activation of NMDA receptors.
DomA	CGNs from Gclm (+/+) and Gclm (–/–) mice	0.01 to 10 µM		Time course (0 to 25 min)		0.1µM domoic acid caused a small and delayed increase (4 fold) in [Ca2+]i, with a full recovery by 20 min.In contrast, the higher concentration of domoic acid (10µM) caused a rapid and robust increase (8 fold) in [Ca2+]i, which was still elevated after 25 min. 0.1µM DA increases [Ca2+]M by about 3 fold, with a delay of about 15 min. In contrast, no changes in [Ca2+]M were observed following 10µM of DA.	Giordano et al., 2007				At the low concentration (0.1µM), the recording of intracellular Ca2+ was antagonized only by NBQX (AMPA receptor antagonist), but not by MK-801 (NMDA receptor antagonist). On the other hand, the higher DA concentration (10µM) caused a rapid and robust increase in intracellular Ca2+ . This effect was antagonized by both NBQX and MK-801, suggesting the importance of NMDA receptors in Ca2+ elevation evoked by DA but only at high doses
DomA	10-13 DIV CGNs obtained from 8-day-old Sprague–Dawley rats	0.1 to 30 µM		Time course (0 to 45 min)		EC50 for DA-induced increase in intracellular Ca2+ was 0.61 µM	Berman et al., 2002				The NMDA receptor antagonist MK-801 significantly reduced both peak and final plateau of intracellular Ca2+ by 30 and 70%, respectively
DomA	Adult male Sprague Dawley rats	10 µM	Brain microinjection			Increased phosphorylated CaMKII and phosphorylated CREB levels	Qiu and Currás-Collazo, 2006				
Glutamate, NMDA	Mouse cortical astrocytes	Glutamate: 100 µM NMDA: 20 µM		Brief (1s application)		Increased intracellular Ca2+ measured through Fluo-3 Flourescence	Palygin et al., 2011				Provides time-series data of intracellular calcium measured through fluoresence given an application of Glu, NMDA, or Glu + D-AP5 in mouse cortical astrocytes. Cells were additionally exposed to D-AP5, an NMDA antagonist, and showed reduced fluorscence changes. (added by DS for AOP 281)
Glutamate	Cultured rat hippocampal	500 µM		Time course (0 to 45		Increased intracellular Ca2+ measured through Fura-2	Michaels and Rothman,				Provides time-series data of intracellular calcium measured through fluoresence, as well as directly providing calculated intracellular calcium concentrations in

	neurons			minutes)		Flourescence	1990				response to high concentrations of applied Glu, both alone and with antagonists. (added by DS for AOP 281)
NMDA, Glutamate	Neocortical neurons of Swiss-Webster mice	Glutamate: 300 µM NMDA: 300 µM		Time course (0 to 20 minutes) and (0 to 2 minutes)		Increased intracellular Ca2+ measured through Fura-2/AM, Fura-2/K+, Fura-2/dextran, BTC	Hyrc et al., 1997				Provides time-series data of intracellular calcium measured through a variety of fluorsence calcium indicators given an application of the selective agonist NMDA. (added by DS for AOP 281)
Glutamate	Computational model (CA1 pyramidal neuron)					Models the concentration of Ca2+ in spine(s) of neuron	Hu et al., 2018				Developed a computational model of a glutamatergic spine which models intracellular calcium dynamics and sources of calcium influx including activation of NMDA receptors. (added by DS for AOP 281)

Glufosinate (GLF)

There are no data showing that an exposure to GLF causes an increase in intra-cellular calcium. Such assumption can be proposed based on a fact that GLF directly activates NMDR as described in the MIE and other relevant KEs of this AOP.

Uncertainties and Inconsistencies

A case of a 59-yr-old woman who ingested a herbicide containing glufosinate was suffering from severe intoxication, however, she did not develop convulsions, which experimentally occurs in rats treated with GLF (Koyama et al., 1994) and is described in other human cases (Watanabe and Sano 1998).

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Relationship: 2783: Status epilepticus leads to Increased, Intracellular Calcium overload

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Intracellular calcium influx has been demonstrated to occur through multiple *in vitro* (Nagarkatti et al., 2010, Pal et al., 1999) and *ex vivo* (Deshpande et al., 2014, Raza et al., 2004) experiments in rat models of status epilepticus.

Key Event Relationship Description

Status epilepticus is defined as continuous seizure activity lasting for more than five minutes, or intermittent seizure activity without regaining of consciousness for the same length of time. Prolonged seizure activity increases neuronal intracellular calcium levels through a variety of mechanisms, such as NMDA receptors, voltage-dependent calcium channels, or release from intracellular calcium stores (Deshpande et al., 2010, Deshpande et al., 2014, Pal et al., 1999).

Evidence Supporting this KER

Biological Plausibility

Calcium influx through voltage-gated channels and ionotropic receptors has been shown to occur in *in vitro* and *in vivo* experiments through targeted antagonism of those channels (Deshpande et al., 2010, Pal et al., 1999).

Empirical Evidence

- Male Sprague-Dawley rats exposed to paraoxon (POX) to induce status epilepticus had increased and prolonged intracellular calcium levels in hippocampal neurons. It appeared that this increase was due to intracellular calcium stores given that inhibition of ryanodine / IP3 receptors lowered calcium levels (Deshpande et al., 2014).
- Status epilepticus induced in male Sprague-Dawley rats in a pilocarpine model showed increases in intracellular hippocampal calcium levels both immediately after status epilepticus and continued to remain elevated days later. Animals that were exposed to pilocarpine but did not develop seizure activity did not show increased intracellular calcium levels (Raza et al., 2004).
- An *in vitro* model of status epilepticus induced by low magnesium in solution with hippocampal cells obtained from 2-day postnatal Sprague-Dawley rats showed increases in intracellular calcium. This was shown to be influx of calcium as reducing extracellular calcium in solution prevented in rise in intracellular calcium (Pal et al., 1999).
- Another *in vitro* model of status epilepticus, using hippocampal neurons cultured from 2-day postnatal Sprague-Dawley rats, induced by low extracellular magnesium showed sustained increases in intracellular calcium (calcium plateau) following three hours of *in vitro* status epilepticus. Calcium levels following SE were reduced when treated with Dantrolene, a ryanodine receptor inhibitor, suggesting the plateau could be due to intracellular calcium stores (Nagarkatti et al., 2010).

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Relationship: 1862: Increased, Intracellular Calcium overload leads to Cell injury/death

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Ca^{2+} cell death is known to occur in both zebrafish and mice (Faria et al., 2015, Choi, 1985).

Key Event Relationship Description

Intracellular calcium (Ca2+) increase can occur from influx through various ion channels (Choi, 1988). Overload of intracellular Ca2+ in the cytoplasm leads to endoplasmic reticulum stress, mitochondrial impairment, and overactivated calcium dependent enzymes such as kinases, phosphatases, proteases, lipases, and endonucleases causing cell damage (Faria et al., 2015, Kaur et al., 2014). Ca2+ elevation occurs shortly (before 1 hour) after exposure to certain toxic compounds (Deshpande et al., 2014).

Evidence Supporting this KER

Biological Plausibility

It is well known that Ca^{2+} signaling overload can trigger cell death mechanisms (Zhivotovsky and Orrenius, 2011). Calcium is also known to partially regulate apoptosis under normal conditions through Ca^{2+} dependent signaling to the mitochondria (Rodrigues et al., 2018).

Empirical Evidence

- Zebrafish models of severe, acute organophosphorus poisoning showed significant calcium signaling pathway changes, characterized by extensive necrosis in the central nervous system. Calcium chelators also reduced the occurrence of this phenotype (Faria et al., 2015).
- Mouse cortical cells showed a decrease in total glutamate-induced cell death when the exposure solution lacked Ca^{2+} (Choi, 1985).
- Rat hippocampal neurons showed a significant positive correlation between an inability to restore resting intracellular calcium concentrations and cell death (Limbrick et al., 1995).
- Cell death was significantly reduced in a low calcium solution in a low Mg^{2+} induced in vitro status epilepticus model of rat hippocampal neurons (Deshpande et al., 2008).
- Neocortical neuron cultures of Swiss-Webster mice exposed to various glutamate receptor agonists showed a correlation between increasing intracellular calcium and increasing LDH release into the medium. Antagonizing NMDA receptors additionally showed both a reduction of intracellular calcium accumulation and LDH release in a dose-dependent manner (Hartley et al., 1993).

Uncertainties and Inconsistencies

Total understanding of the complex signaling involved with intracellular Ca^{2+} has not been fully explored, but there is plenty of evidence supporting the link between Ca^{2+} and cell death (Nagarkatti et al., 2009). There is also evidence that the pathway of increased Ca^{2+} makes a difference in the neurotoxicity of the Ca^{2+} influx, showing NMDAR mediated influx is more lethal compared to other Ca^{2+} channels (Lau and Tymianski, 2010).

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Relationship: 364: Cell injury/death leads to N/A, Neurodegeneration

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	Moderate	
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Unspecific	High

Neurodegeneration from cell death is widely accepted, neurodegenerative models have used various species including mice and zebrafish for different neurodegenerative diseases (Dawson et al., 2018)

Information Specific to DomA

There is an overall agreement regarding the histopathology of the brain lesions related to acute DomA neurotoxicity across certain species. Data

derived from humans, rodents, non-human primates and sea lions suggest that common neurodegeneration features in selected brain areas are found despite the fact that study design, estimated exposure, processing of samples and history of event may differ (Pulido, 2008).

Furthermore, the distribution of brain damage by DomA has also been established by magnetic resonance imaging microscopy (MRM) for both human and rat, demonstrating similar distribution as that described by histopathological studies (Pulido, 2008).

It is important to notice that human sensitivity to DomA exposure is well documented in the published literature and seems to be much higher than in other species (Lefebvre and Robertson 210; Barlow et al., 2004). In 1987 in Canada, more than 200 people became acutely ill after ingesting mussels contaminated with DomA. The outbreak resulted in 20 hospitalizations and four deaths. Clinical effects observed included gastrointestinal symptoms and neurotoxic effects such as hallucinations, memory loss and coma. For this reason, the condition was termed amnesic shellfish poisoning (Barlow et al., 2004). The neurotoxic properties of DomA result in neuronal degeneration and necrosis in specific regions of the hippocampus (Teitelbaum et al., 1990).

Key Event Relationship Description

Cell death of neurons directly causes neurodegeneration characterized by abnormal neuronal loss (Przedborski et al., 2003). While the upstream event is unspecific as to the type of cell affected, neurodegeneration is caused by cell death in neurons specifically.

Evidence Supporting this KER

Biological Plausibility

There is well established mechanistic understanding supporting the relationship between these two KEs.

Neurodegeneration in the strict sense of the word, is referring to any pathological condition primarily affecting brain cell populations (Przedborski et al., 2003). At the histopathological level, neurodegenerative conditions are described by neuronal death and reactive gliosis (Przedborski et al., 2003).

Empirical Evidence

Include consideration of temporal concordance here

Evidence applicable to domoic acid (DomA):

- Acute brain damage induced by DomA is characterized by neurodegenerative changes consisting of neuronal shrinkage, vacuolization of the cytoplasm, cell drop out, edema, microvacuolation of the neuropil and hydropic cytoplasmic swelling of resident astrocytes (reviewed in Pulido et al., 2008). These histopathological changes can be identified within structures of the limbic system, in hippocampus, in the CA3, CA4 or hilus of the dentate gyrus (DG) (reviewed in Pulido et al., 2008). Other brain areas known to be affected by DomA include: the olfactory bulb, the piriform and entorhinal cortices, the lateral septum, the subiculum, the arcuate nucleus and several amygdaloid nuclei. The area postrema is another target for DomA toxicity as it has been identified in both rodents and non-human primates, providing a possible explanation of emetic symptoms (nausea, retching, and/or vomiting) induced by DomA. There has been an effort to map and create a 3-D reconstruction of DomA-induced neurodegeneration in the mouse brain demonstrating that the affected areas include the olfactory bulb, septal areas and the limbic system (Colman et al., 2005; Barlow et al., 2004).
- Female Sprague-Dawley rats dosed once intraperitoneally (i.p.) with 0, 1, 2, 4, or 7.5 DomA mg /kg of body weight were euthanized after 24 h and their nervous system was examined for microscopic alterations revealing neuronal degeneration and vacuolation of the neurophil in the limbic and the olfactory systems (Tryphonas et al., 1990).
- The mean of TUNEL positive cells in the hippocampus was increased (6-fold) in mice injected i.p. at a dose of 2 DomA mg/kg once a day for 3 weeks (Lu et al., 2012). However, the same treatment protocol did not cause any neurodegeneration (Lu et al., 2012). In contrast, when the same treatment was prolonged for one more week (total 4 weeks), the mean values of NeuN-positive cells in the hippocampal CA1 sections of DomA-treated cells decreased by 3 fold compared to controls (Lu et al., 2012). This study showed that the incidence of upstream KE (cell death) was higher than the incidence of downstream KE (neurodegeneration) and that upstream KE (cell death) preceded downstream KE (neurodegeneration).
- The bcl-2 and bax mRNA levels in the hippocampus were significantly increased at 16 h and gradually decreased at 24 h following the administration of DomA (0.75 mg/kg body weight) in adult rats. In situ hybridization analysis revealed complete loss of bcl-2, bax, and caspase-3 mRNA at 24 h after DA administration in the region of the hippocampus, whereas neurodegeneration by Nissl staining was detected at the same time point but was more pronounced after 5 days (Ananth et al., 2001). This study showed that both KEs occurred after exposure to the same dose of DomA and that the upstream KE (cell death) occurred earlier than the downstream KE (neurodegeneration).
- Adult rats received i.p. injections with DomA 1.0 mg/kg/h until animals exhibited first motor seizures. After a week of recovery, aggressive behaviors and motor seizures of the animals had been monitored for 3h twice a week. After 12 weeks, animals were euthanized and brains were examined for indications of cell loss by using thionine (Nissl) staining, which highlights the cell bodies of all living neurons. In piriform cortex a reduced cell density was noted in the medial layer 3 (1.3-1.8 fold decrease compared to controls), an area that shows also prominent amino cupric staining (stain that assesses neuronal damage) (Tiedeken and Ramsdell, 2013a). The same research group reported that by following the above experimental procedure but sacrificing the rats 7 days after DomA-induced seizures intense and widespread silver reaction product in the olfactory bulb occurred, whereas minor or no evident damage was found in the hippocampus (Tiedeken et al., 2013b).
- Intraperitoneal injection of DomA 0.5 mg/kg to adult C57BL/6 male mice resulted in loss of 32% and 30% of Nissl-stained neurons in hilus and CA1 pyramidal layer of the hippocampus, respectively, compared to control mice 7 d after the administration (Antequera et al., 2012).
- The severity and extent of hippocampal neuronal degeneration varied significantly depending on the dose of DomA (1 µM to 1 mM) that was tested after microinjection to adult male Sprague Dawley rats (Qiu and Currás-Collazo, 2006). In rats dosed with 1 mM DomA and sacrificed after 24 h, histopathological analysis using toluidine blue staining revealed extensive neuronal damage throughout the ipsilateral hippocampal structure. Shrunken, disorganized and densely stained neurons of irregular shape were identified throughout CA1, CA2, CA3 pyramidal layer as well as the dentate gyrus hilus and granule cells layer. For the 100 µM group animals, CA1 neuronal changes were less prominent, whereas 10 µM and 1 µM DomA did not produced any resolvable histopathological changes (Qiu and Currás-Collazo, 2006).
- Adult male rats treated with 2 mg/kg DomA i.p. were sacrificed after 3 d and showed that the silver stain used to assess neurodegeneration clearly distinguished treated from control animals, whereas a number of other markers failed to do so (Scallet et al., 2005). The same results were found after even longer exposure times (7 d) to DomA (Appel et al., 1997).
- Male Wistar rats were given a single i.v. injection of DA (0.75 mg/kg) in the right external jugular vein and brain sections were stained with Nissl stain at 5 d

after DomA administration. Histopathological analysis revealed a large number of darkly stained shrunken neurons in the hippocampus (Ananth et al., 2003). However, complete absence of hippocampal neurons was observed in CA1 and CA3 regions in DomA treated animals at 3 months after DomA administration (Ananth et al., 2003).

- In 2-3 week old hippocampal slice cultures, derived from 7 day old rat pups, DomA (0.1-100 µM) was added to the culture medium and neurodegeneration in the fascia dentata (FD), CA3 and CA1 hippocampal subfields was measured. The CA1 region appeared to be most sensitive to DomA, with an EC50 value of 6 µM DomA after estimating the PI-uptake at 72 h (Jakobsen et al., 2002).
- Cynomolgus monkeys were given i.v. a range of DomA doses from 0.25 to 4.0 mg/kg. Silver staining of brain sections revealed that doses in the range of 0.5-1.0 mg/kg produced a small area of silver grains restricted to axons of the hippocampal CA2 stratum lucidum, whereas higher concentrations produced degenerating axons and cell bodies (Slikker et al., 1998). The same research group treated i.v. adult monkeys with DomA at one of a range of doses from 0.25 to 4 mg/kg. After a week, silver staining demonstrated degenerating axons and cell bodies that was mild and restricted to CA2 stratum lucidum at a lower doses (0.5 to 1.0 DomA mg/kg). Doses of more than 1.0 mg/kg caused widespread damage to pyramidal neurons and axon terminals of CA4, CA3, CA2, CA1, and subiculum subfields of the hippocampus. However, when DomA was orally administered to cynomolgus monkeys at doses of 0.5 mg/kg for 15 days and then at 0.75 mg/kg for another 15 days no histopathoogical changes in the brain were detected (Truelove et al., 1997).
- In humans, autopsy of individuals intoxicated by DomA revealed brain damage characterized by neuronal necrosis and in the hippocampus and the amygdaloid nucleus (Pulido, 2008). The thalamus and subfrontal cortex were damaged only in some patients suffering from Amnesic Shellfish Poisoning (ASP). The detailed examination of one patient intoxicated by DomA revealed complete neuronal loss in the CA1, CA3 and CA4 regions, whereas moderate loss was seen in the CA2 region (Cendes et al., 1995). Non-severe neuronal loss was detected in amygdale, overlying cortex, the dorsal and ventral septal nuclei, the secondary olfactory areas, and the nucleus accumbens (Cendes et al., 1995).

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Cell death (KE up) (measurements, quantitative if available)	Neurodegeneration (KE down) (measurements, quantitative if available)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Female Sprague-Dawley rats	0, 1, 2, 4, or 7.5 DomA mg /kg	intraperitoneally (i.p.)	Euthanized after 24 h		Neuronal degeneration and vacuolation of the neuropil in the limbic and the olfactory systems	Tryphonas et al., 1990				
DomA	16-month-old male ICR mice	2 mg/kg	Intraperitoneally (i.p.)	Once a day for 3 or 4 weeks	The mean of TUNEL positive cells in the hippocampus was increased (6 fold). The levels of bcl-2, procaspase-3 and procaspase-12 were significantly decreased and the activation of caspase-3 and caspase-12 in the mouse hippocampus were increased.	The mean OD of NeuN immunoreactivity in the hippocampus of mice decreased (3 fold) indicating significant neuron loss by apoptosis, which is one of the pathological hallmarks of neurodegeneration	Lu et al., 2012	Upstream KE (cell death) precedes downstream KE (neurodegeneration)	Same dose	Incidence of upstream KE (cell death) is higher than the incidence of downsteam KE (neurodegeneration)	Mice treated with DomA once a day for 3 weeks showed that apoptosis was increased. However, the same treatment protocol did not cause any neurodegeneration. In contrast, when the same treatment was prolonged for one more week (total 4 weeks) induced marked neuron loss.
DomA	Adult rats	0.75 mg/kg	intravenously (i.v.)	Euthanized after 2, 5, 14, or 21 days	The bcl-2 and bax mRNA levels in the hippocampus were significantly increased at 16 h and gradually decreased at 24 h following the administration of DomA. In situ hybridization analysis revealed complete loss of bcl-2, bax, and caspase-3 mRNA at 24 h after DomA administration in the region of hippocampus.	Neurodegeneration by Nissl staining was detected at the same time point but was reported to be more pronounced after 5 days	Ananth et al., 2001	Upstream KE (cell death) occurs earlier that downstream KE (neurodegeneration).	Same dose		
DomA	Adult rats	1.0 mg/kg/h until animals exhibited first motor seizures	i.p.	Euthanized after 12 weeks		In piriform cortex a reduced cell density was noted in the medial layer 3 (1.3-1.8 fold decrease compared to controls), an area that showed also prominent amino cupric staining (stain that assesses neuronal damage).	Tiedeken and Ramsdell, 2013a				
		1.0 mg/kg/h until				Intense and widespread silver reaction product in					

DomA	Adult rats	animals exhibited first motor seizures	i.p.	Euthanized after 1 week		the olfactory bulb, whereas minor or no evident damage was found in hippocampus.	Tiedeken et al., 2013b				
DomA	Adult C57BL/6 male mice	0.5 mg/kg	i.p.	Euthanized after 1 week		DomA treatment resulted in the loss of 32% and 30% of Nissl-stained neurons in hilus and CA1 pyramidal layer of the hippocampus, respectively, compared to control mice.	Antequera et al., 2012				
DomA	Adult male Sprague Dawley rats	1 µM to 1 mM	microinjection	Euthanized after 24 h		In rats dosed with 1 mM DomA and sacrificed after 24 h, histopathological analysis using toluidine blue staining revealed extensive neuronal damage throughout the ipsilateral hippocampal structure. Shrunken, disorganized and densely stained neurons of irregular shape were identified throughout CA1, CA2, CA3 pyramidal layer as well as the dentate gyrus hilus and granule cells layer. For the 100 µM group animals, CA1 neuronal changes were less prominent, whereas 10 µM and 1 µM DomA did not produce resolvable histopathological changes.	Qiu and Currás-Collazo, 2006				
DomA	Adult male rats	2 mg/kg	i.p.	Euthanized after 3 or 7 days		DA treatment for 3 d showed that the silver stain that was used to assess neurodegeneration clearly distinguished treated from control animals , the same was true for longer exposure time (7 d).	Scallet et al., 2005, Appel et al., 1997				
DomA	Male Wistar rats	0.75 mg/kg	i.v.	Euthanized after 5 days or 3 months		Histopathological analysis revealed a large number of darkly stained shrunken neurons in the hippocampus However, complete absence of hippocampal neurons was observed in CA1 and CA3 regions in DA treated animals at 3 months after DomA administration.	Ananth et al., 2003				
DomA	2-3 week old rat hippocampal slice cultures, derived from 7 day old rat pups	0.1-100 µM		72 h		DomA induced neurodegeneration in the fascia dentata (FD), CA3 and CA1 hippocampal subfields. The CA1 region appeared to be most sensitive to DomA, with an EC50 value of 6 µM DomA, estimated from the PI-uptake at 72 h .	Jakobsen et al., 2002				
						Silver staining of brain sections revealed that doses in the range of 0.5-1.0 mg/kg produce a small area of silver grains restricted to axons of the hippocampal CA2 stratum lucidum,					

DomA	Cynomolgus monkeys	0.25 to 4.0 mg/kg	i.v.	Euthanized after 1 week		whereas higher concentrations revealed degenerating axons and cell bodies. After a week, silver staining demonstrated degenerating axons and cell bodies that was mild and restricted to CA2 stratum lucidum at the lower doses (0.5 to 1.0 DomA mg/kg). Doses of more than 1.0 mg/kg caused widespread damage to pyramidal neurons and axon terminals of CA4, CA3, CA2, CA1, and subiculum subfields of the hippocampus.	Slikker et al., 1998, Truelove et al., 1997				

Gap of knowledge: there are no studies showing that glufosinate (GLF)-induced cell death leads to neurodegeneration.

Evidence applicable to acetylcholinesterase inhibition:

Experiments using the nerve agent soman, an acetylcholinesterase inhibitor, showed major changes in various areas of the brain including the cerebral cortex, piriform cortex, amygdala, hippocampus, thalamus and striatum from neuronal lesions (Acon-Chen et al., 2016).

Uncertainties and Inconsistencies

There are various methods to categorize neurodegeneration from cell death, and there are “different clinical pictures” depending on the area or areas of the brain affected (Przedborski et al., 2003).

Domoic acid considerations:

Zebrafish were exposed for 36-weeks to DomA and showed no excitotoxic neuronal death and no histopathological lesions in glutamate-rich brain areas (Hiolski et al., 2014).

Administration of DomA (9.0 mg DomA kg(-1) bw, i.p.) to seabream (*Sparus aurata*) lead to measurement of 0.61, 0.96, and 0.36 mg DomA kg(-1) of brain tissue at 1, 2 and 4 hours. At this dose but also at lower concentrations (0.45 and 0.9 mg DomA kg(-1) bw) no major permanent brain damage was detected (Nogueira et al., 2010). Leopard sharks possess the molecular target for DomA but it has been shown to be resistant to doses of DomA that can cause neurotoxicity to other vertebrates, suggesting the presence of some protective mechanism (Schaffer et al., 2006).

All these reports suggest species specific susceptibility to DomA toxicity.

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