

AOP ID and Title:

SNAPSHOT

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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

Short Title: Binding of antagonist to NMDARs can lead to neuroinflammation and neurodegeneration

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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	EAGMST Under Review	1.13	Included in OECD work plan

Abstract

This AOP is an extension of AOP 13 linking NMDAR chronic inhibition during brain development to impairment of learning and memory. It links chronic NMDA receptors inhibition during brain development to Adverse Outcomes, i.e. neurodegeneration in hippocampus and cortex with amyloid plaque deposition and tau hyperphosphorylation and impairment of learning and memory, which are considered as hallmark of Alzheimer's disease. It introduces another KE, Neuroinflammation, which is involved in several neurodegenerative diseases. With Neuroinflammation and Neurodegeneration, this AOP connects to AOP 48, where in adult brain, « neuroinflammation » leads to « Neurodegeneration » ; « Neurodegeneration » leads to « Decreased neuronal network function », which finally leads to « Impairment of learning and memory ». Both neurodegeneration and cognitive deficits are observed in Alzheimer's pathology. But as neurodegenerative diseases are complex and multifactorial, the authors proposed two Adverse outcomes: one at the organism level « Impairment of learning and memory», and one at the organ level, « neurodegeneration ». Both are regulatory endpoints. This AOP integrates in the network of AOPs relative to neurotoxicity testing.

This AOP is based on the hypothesis of Landrigan and coworkers (2005) proposing an early origin of neurodegenerative diseases in later life. The chemical initiator known to block NMDARs and used in this AOP for the empirical support is lead (Pb), which is a well-known developmental neurotoxicant. In epidemiological studies of adults, cumulative lifetime lead exposure has been associated with accelerated decline in cognition (Bakulski et al., 2012), suggesting that long term exposure to lead during brain development or occupational exposure in adulthood increases the risk to develop a neurodegenerative disease of Alzheimer's type. The long latency period between exposure and late-onset of neurodegeneration and cognitive deficits gives a very broad life stage applicability, where developmental exposure has consequences in the aging brain. Such a long temporal delay between exposure and adverse outcome is a real difficulty and challenge for neurotoxicity testing. As the Key Event « Neuroinflammation » appears to play a crucial role in the neurodegenerative process, the authors proposed to include the measurement of this apical KE in the battery of regulation-required neurotoxicity testing.

The recent review by Tartaglione and coworkers (2016) is a very good summary of the challenges and experimental studies described in this AOP.

Summary of the AOP

Stressors

Name	Evidence
Lead	

Molecular Initiating Event

Title	Short name
Binding of antagonist, NMDA receptors (https://aopwiki.org/events/201)	Binding of antagonist, NMDA receptors

201: Binding of antagonist, NMDA receptors (<https://aopwiki.org/events/201>)

Short Name: Binding of antagonist, NMDA receptors

AOPs Including This Key Event

AOP ID and Name	Event Type
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	MolecularInitiatingEvent
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 (https://aopwiki.org/aops/12)	MolecularInitiatingEvent

Stressors

Name
Lead

Biological Organization

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Glu and glycine are endogenous agonists that bind to LBD of specific NMDA receptor subunits. In this binding site numerous competitive exogenous antagonists have been identified to cause closure of binding site and inhibition of NMDA receptor (reviewed in Traynelis et al., 2010). Here, are listed some known competitive antagonists for NMDA receptor, some of them are specific to NR1 subunit and some to NR2 subunit:

α-AA, α-amino adipate;

5,7-DCKA, 5,7-dichlorokynurenic acid;

7-CKA, 7-chlorokynurenic acid;

ACEA-1011, 5-chloro-7-trifluoromethyl-1,4-dihydro-2,3-quinoxalinedione;

ACEA-1021, licostinel;
 AP5, 2-amino-5-phosphonopentanoate;
 AP7, 2-amino-7-phosphonopentanoate;
 CGP-61594, (\pm)-trans-4-[2-(4-azidophenyl)acetylamino]-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-carboxylic acid;
 CGP-40116, d-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid;
 CGP-43487, d-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid methyl ester;
 CGP-58411, 7-chloro-4-hydroxy-3-phenyl-1H-quinolin-2-one;
 CGS-19755, (2R,4S)-4-(phosphonomethyl)piperidine-2-carboxylic acid;
 CPP, 4-(3-phosphonopropyl) piperazine-2-carboxylic acid;
 GV150,526A, gavestinel;
 GV196,771A, (E)-4,6-dichloro-3-[(2-oxo-1-phenyl-3-pyrrolidinylidene)methyl]-1H-indole-2-carboxylic acid;
 L-689,560, 4-trans-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline;
 L-701,324, 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolone;
 MDL105,519, (E)-3-(2-phenyl-2-carboxyethenyl)-4, 6-dichloro-1H-indole-2-carboxylic acid;
 PBPD, (2S,3R)-1-(biphenyl-4-carbonyl)piperazine-2,3-dicarboxylic acid;
 PMPA, (R,S)-4-(phosphonomethyl)-piperazine-2-carboxylic acid;
 PPDA, (2S,3R)-1-(phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid

Besides competitive antagonists, noncompetitive antagonists have also been designed like phenylethanolamine ifenprodil that interacts with the NR2B extracellular amino-terminal domain. It has been suggested that they act by stabilizing an agonist-bound state in which the receptor has a low open probability. Other more potent derivatives of ifenprodil are: α -(4-hydroxyphenyl)- β -methyl-4-(phenylmethyl)-1-piperidine propanol (Ro 25-6981), 1-[2-(4-hydroxy-phenoxy)-ethyl]-4-(4-methyl-benzyl)-piperidin-4-ol (Ro 63-1908), besonprodil (CI-1041), and traxoprodil mesylate (CP-101,606). Ethanol has been proposed to be a noncompetitive antagonist of NMDA receptors, binding to NR2 subunit (Nagy, 2008). Inhibition of NMDA receptor function by ethanol and interactions between ethanol and the noncompetitive NMDA receptor antagonist ifenprodil have been examined in neocortical neurons from rat and human embryonic kidney (HEK) 293 cells expressing recombinant NMDA receptors (Lovinger, 1995). Recently, a structural model has been suggested that predicts the presence of four sites of ethanol action on the NMDA receptor, each containing four pairs of positions in the NR1/NR2 subunits (reviewed in Chandrasekar, 2013). Some other antagonists can become trapped in the pore of the NMDA receptor after channel closure and these antagonists are called uncompetitive or trapping blockers. The most well studied NMDA receptor uncompetitive antagonists are Mg²⁺, polyamines, phencyclidine, ketamine, MK-801, memantine, amantadine, pentamidine, 9-tetrahydroaminoacridine, dextromethorphan, and its metabolite dextrorphan. MK-801 has been shown to prevent toluene-induced alterations in pattern-elicited visual-evoked potentials in vivo, suggesting the possibility that the binding site of toluene might be common with the one of MK-801 (Bale et al., 2007). However, another study suggests that toluene interference with the NMDA receptor might not be exclusively because of the binding to the channel pore (Smothers and Woodward, 2007) but it may involve some other binding sites. Lead (Pb²⁺) is considered a voltage independent antagonist of NMDA receptors and it is believed that possibly shares the same binding site with Zn²⁺ (reviewed in Neal and Guilarte, 2010; Traynelis et al., 2010). However, studies done in recombinant NR2A- and NR2B- containing NMDA receptors with mutated Zn²⁺ binding sites exhibit that additional structural elements, different from those important for Zn²⁺ binding are involved in Pb²⁺ binding site (reviewed in Neal and Guilarte, 2010). Similarly, there are contradicting experimental evidence and disagreement about Pb²⁺'s role as competitive or non-competitive antagonist (Neal and Guilarte, 2010).

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

The evolution of NMDAR subunits (NR1, NR2, NR3) is well-conserved throughout different species from lower organism to mammals, including humans (Ewald and Cline, 2009; Tikhonov and Magazanik, 2009; Koo and Hampson, 2010; Teng et al., 2010; Flores-soto et al., 2012).

Many of the binding sites for the noncompetitive or competitive antagonists e.g. for binding of dizocilpine (MK-801), phencyclidine, D-2-amino-5-phosphonopentanoate (AP5) and 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (R-CPP) are also conserved in *Drosophila* (reviewed in Xia and Chiang, 2009).

Cellular membranes can be prepared from different brain areas of distinct species. Using [³H]MK-801, high affinity binding sites for MK-801 were detected in membranes of the rat brain (Woodruff et al., 1987). The same binding assay has been used in preparations from human brains mostly by patients with neurodegenerative disorders (Slater et al., 1993) as well as from different marine, avian species (Scheuhammer et al., 2008) and insects (Eldefrawi et al., 1993).

How this Key Event Works

Biological state: L-glutamate (Glu) is a neurotransmitter with important role in the regulation of brain development and maturation processes. Two major classes of Glu receptors, ionotropic and metabotropic, have been identified. Due to its physiological and pharmacological properties, Glu activates three classes of ionotropic receptors named: α -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA receptors), 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate receptors) and N-methyl-D-aspartate (NMDA receptors, NMDARs), which transduce the postsynaptic signal. Ionotropic glutamate receptors are integral membrane proteins formed by four large subunits that compose a central ion channel pore. In case of NMDA receptors, two NR1 subunits are combined with either two NR2 (NR2A, NR2B, NR2C, NR2D) subunits and less commonly are assembled together with a combination of NR2 and NR3 (A, B) subunits (reviewed in Traynelis et al., 2010). To be activated NMDA receptors require simultaneous binding of both glutamate to NR2 subunits and of glycine to either NR1 or NR3 subunits that provide the specific binding sites named extracellular ligand-binding domains (LBDs). Apart from LBDs, NMDA receptor subunits contain three more domains that are considered semiautonomous: 1) the extracellular amino-terminal domain that plays important role in assembly and trafficking of these receptors; 2) the transmembrane domain that is linked with LBD and contributes to the formation of the core of the ion channel and 3) the intracellular carboxyl-terminal domain that influences membrane targeting, stabilization, degradation and post-translation modifications.

Biological compartments: The genes of the NMDAR subunits are expressed in various tissues and are not only restricted to the nervous system. The level of expression of these receptors in neuronal and non-neuronal cells depends on: transcription, chromatin remodelling, mRNA levels, translation, stabilization of the protein, receptor assembly and trafficking, energy metabolism and numerous environmental stimuli (reviewed in Traynelis et al., 2010).

In hippocampus region of the brain, NR2A and NR2B are the most abundant NR2 family subunits. NR2A-containing NMDARs are mostly expressed synaptically, while NR2B-containing NMDARs are found both synaptically and extrasynaptically (Tovar and Westbrook, 1999).

General role in biology: NMDA receptors, when compared to the other Glu receptors, are characterized by higher affinity for Glu, slower activation and desensitisation kinetics, higher permeability for calcium (Ca²⁺) and susceptibility to potential-dependent blockage by magnesium ions (Mg²⁺). NMDA receptors are involved in fast excitatory synaptic transmission and neuronal plasticity in the central nervous system (CNS). Functions of NMDA receptors:

1. They are involved in cell signalling events converting environmental stimuli to genetic changes by regulating gene transcription and epigenetic modifications in neuronal cells (Cohen and Greenberg, 2008).
2. In NMDA receptors, the ion channel is blocked by extracellular Mg²⁺ and Zn²⁺ ions, allowing the flow of Na⁺ and Ca²⁺ ions into the cell and K⁺ out of the cell which is voltage-dependent. Ca²⁺ flux through the NMDA receptor is considered to play a critical role in pre- and post-synaptic plasticity, a cellular mechanism important for learning and memory (Barria and Malinow, 2002).
3. The NMDA receptors have been shown to play an essential role in the strengthening of synapses and neuronal differentiation, through long-term potentiation (LTP), and the weakening of synapses, through long-term depression (LTD). All these processes are implicated in the memory and learning function (Barria and Malinow, 2002).

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? There is no OECD advised method for measuring NMDA receptor binding of antagonists. However, there are methods described in the scientific literature that allow measuring:

1. Ex vivo: The most common assay used is the NMDA receptor (MK801 site) radioligand competition binding assays (Reynolds, 2001; Gao et al., 2013; <http://pdsp.med.unc.edu/UNC-CH%20Protocol%20Book.pdf>; <http://pdsp.med.unc.edu/UNC-CH%20Protocol%20Book.pdf>); <http://www.currentprotocols.com/WileyCDA/CPUnit/refId-ph0120.html> (<http://www.currentprotocols.com/WileyCDA/CPUnit/refId-ph0120.html>). This assay is based on the use of the most potent and specific antagonist of this receptor, MK801 that is used to detect and differentiate agonists and antagonists (competitive and non-competitive) that bind to this specific site of the receptor. Also radioligand competition binding assay can be performed using D, L-(E)-2-amino-4-[3H]-propyl-5-phosphono-3-pentenoic acid ([3H]-CGP 39653), a high affinity selective antagonist at the glutamate site of NMDA receptor, which is a quantitative autoradiography technique (Mugnaini et al., 1996). D-AP5, a selective N-methyl-D-aspartate (NMDA) receptor antagonist that competitively inhibits the glutamate binding site of NMDA receptors, can be studied by evoked electrical activity measurements. AP5 has been widely used to study the activity of NMDA receptors particularly with regard to researching synaptic plasticity, learning, and memory (Evans et al., 1982; Morris, 1989).

2. In silico: The prediction of NMDA receptor targeting is achievable by combining database mining, molecular docking, structure-based pharmacophore searching, and chemical similarity searching methods together (Korkut and Varnali, 2003; Koutsoukos et al., 2011; Gao et al., 2013; Mazumber and Borah, 2014; Chtitaa et al., 2015).

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Key Events

Title	Short name
Inhibition, NMDARs (https://aopwiki.org/events/195)	Inhibition, NMDARs
Decreased, Calcium influx (https://aopwiki.org/events/52)	Decreased, Calcium influx
Reduced, Release of BDNF (https://aopwiki.org/events/381)	Reduced, Release of BDNF
N/A, Cell injury/death (https://aopwiki.org/events/55)	N/A, Cell injury/death
N/A, Neuroinflammation (https://aopwiki.org/events/188)	N/A, Neuroinflammation

195: Inhibition, NMDARs (<https://aopwiki.org/events/195>)

Short Name: Inhibition, NMDARs

AOPs Including This Key Event

AOP ID and Name	Event Type
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 (https://aopwiki.org/aops/12)	KeyEvent
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Biological Organization

Level of Biological Organization
Molecular

Evidence Supporting Applicability of this Event

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human	<i>Homo sapiens</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	<i>Mus musculus</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

The cellular expression of the NMDAR subunits has been studied in both adult human cortex and hippocampus (Scherzer et al., 1998) as well as during the development of the human hippocampal formation (Law et al., 2003). The whole-cell patch clamp recording techniques have been used in NMDA receptors expressed in human TsA cells (derivative of the human embryonic kidney cell line HEK-293) (Ludolph et al., 2010). Cell-attached single-channel recordings of NMDA channels has been carried out in human dentate gyrus granule cells acutely dissociated from slices prepared from hippocampi surgically removed from human patients (Lieberman and Mody, 1999).

It is important to note that in invertebrates the glutamatergic synaptic transmission has inhibitory and not 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).

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2. In NMDA receptors, the ion channel is blocked by extracellular Mg²⁺ and Zn²⁺ ions, allowing the flow of Na⁺ and Ca²⁺ ions into the cell and K⁺ out of the cell which is voltage-dependent. Ca²⁺ flux through the NMDA receptor is considered to play a critical role in pre- and post-synaptic plasticity, a cellular mechanism important for learning and memory (Barria and Malinow, 2002).
3. The NMDA receptors have been shown to play an essential role in the strengthening of synapses and neuronal differentiation, through long-term potentiation (LTP), and the weakening of synapses, through long-term depression (LTD). All these processes are implicated in the memory and learning function (Barria and Malinow, 2002).

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 close state, respectively.

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 (Ogdon and Stanfield, 2009; Zhao et al., 2009).

Microelectrode array (MEA) recordings are used to measure electrical activity in cultured neurons in response to NMDA receptor activation or inactivation (Keefer et al., 2001, Gramowski et al., 2000 and Gopal, 2003; Johnstone et al., 2010). MEAs can also be applied in higher throughput platforms to facilitate screening of numerous chemical compounds based on electrical activity measurements (McConnell et al., 2012).

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52: Decreased, Calcium influx (<https://aopwiki.org/events/52>)

Short Name: Decreased, Calcium influx

AOPs Including This Key Event

AOP ID and Name	Event Type
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	KeyEvent
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 (https://aopwiki.org/aops/12)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mice	Mus sp.	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)
zebrafish	Danio rerio	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

Ca²⁺ homeostatic system is known to be highly conserved throughout evolution and is present from humans to invertebrates (Case et al., 2007).

How this Key Event Works

Biological state: Under physiological resting conditions of the cell, the free intracellular Ca²⁺ reaches around 100 nM, whereas the extracellular Ca²⁺ can be found at higher concentrations of 1.2 mM that under certain stimulus may invade the cell (Berridge et al, 2000). Six to seven oxygen atoms surround Ca²⁺, whereas the protein chelator of Ca²⁺ is the EF motif that is present in many proteins such as calmodulin (Clapham, 2007). The EF-hand is a helix-loop-helix calcium-binding motif in which two helices pack together at an angle of approximately 90 degrees (Lewit-Bentley and Réty, 2000). The two helices are separated by a loop region where calcium actually binds. The EF notation for the motif is derived from the notation applied to the structure of parvalbumin, in which the E and F helices were originally identified as forming this calcium-binding motif.

Biological compartments: Ca²⁺ ions accumulate in the cytoplasm, cellular organelles (e.g. mitochondria and endoplasmic reticulum) and nucleus in response to diverse classes of stimuli.

General role in biology: In order to adapt to altered stimulus from exposure to different environmental factors, cells require signal transmission. However, signalling needs messengers whose concentration is modified upon stimulus (Clapham, 2007). Ca²⁺ ions act as an important intracellular messenger playing the role of ubiquitous signalling molecules and consequently regulate many different cellular functions (Berridge, 2012; Hagenston and Bading, 2011). Given its important role in processes that are fundamental to all cell types, Ca²⁺ homeostasis is tightly regulated by intracellular and extracellular mechanisms (Barhoumi et al., 2010). Intracellular Ca²⁺ concentration is regulated by opening or closing channels in the plasma membrane. Additionally, the Ca²⁺ ions can be released from intracellular stores of the endoplasmic reticulum (ER) through ryanodine receptors (RyRs) or inositol 1,4,5-trisphosphate receptors (InsP₃Rs). Ca²⁺ homeostasis is also regulated by the mechanisms that remove Ca²⁺ from the cytosol, for example pumps in both cell membrane and ER membrane. In addition, cytosolic Ca²⁺ regulation involves accumulation of Ca²⁺ in mitochondria that have the capacity to buffer the excess of cytoplasmic Ca²⁺ ions. In neurons, Ca²⁺ ions regulate many critical functions. Firstly, they contribute to dendritic electrical signalling, producing postsynaptic depolarization by the current carried by Ca²⁺ ions. Secondly, Ca²⁺ activates Ca²⁺-sensitive proteins such as different kinases, calcineurin and calpain, triggering signalling pathways critical for cell physiology. Modification of the gene transcription is the final outcome of the Ca²⁺ ions impact on long-term modifications affecting neurotransmitters release (reviewed in Neher and Sakaba, 2008), neuronal differentiation, synapse function and cell viability (Clapham, 2007; Higley and Sabatini, 2012). Thus, the Ca²⁺ that enters and accumulates in cytoplasm and nucleus is a central signalling molecule that regulates synapse and neuronal cell function, including learning and memory processes (Berridge, 2012; Hagenston and Bading, 2011).

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 method is available to measure intracellular Ca²⁺.

The gold standard method for measuring Ca²⁺ current through NMDA receptor is patch clamp electrophysiology (Blanke and VanDongen, 2009).

In vitro, well-established flow cytometric or high content imaging analysis with specific fluorescent dyes (Ca²⁺-sensitive fluorophores) such as Fura-2, Oregon Green-BAPTA, Fluo-4 and X-Rhod exist for determination of intracellular Ca²⁺ concentration. The use of different fluorometric calcium indicators in neuroscience and neurotoxicology have been recently reviewed by Grienberger and Konnerth (2012) and Calvo et al (2015).

Barhoumi et al. 2010 summarised all the methods to measure cytosolic Ca²⁺ alterations due to exposure to neurotoxic compounds, including steady state, short-term kinetic measurements of stimulated Ca²⁺ transients

and dynamic measurements. This paper further discusses the strengths and weaknesses of each approach in intracellular Ca²⁺ measurements and its applicability in high throughput screening.

For quantitative estimation of Ca²⁺ in dendritic spines, besides of Ca²⁺-sensitive fluorophores the use of two-photon released caged neurotransmitters has been suggested as it allows direct stimulation of visualized spines (Higley and Sabatini, 2012). In Higley and Sabatini 2012 further technical information can be found in relation to study Ca²⁺ in dendritic spines.

Furthermore, there are three methods for measuring Ca²⁺ influx in NMDA receptors that involve the measurement of 1) relative Ca²⁺ permeability, 2) channel blockage by Ca²⁺, and 3) fractional Ca²⁺ currents from whole-cell currents determined in the presence of high concentrations of intracellular Fura-2 (Traynelis et al., 2010).

In vivo, two-photon Ca²⁺ imaging using Ca²⁺-sensitive fluorescent indicators that measure changes in intracellular Ca²⁺ concentration as a readout for suprathreshold and subthreshold neuronal activity has also been used to study learning and memory in live rodents (Chen et al., 2013) The last two decades the neuronal function of the larval and adult zebrafish has been extensively studied using Ca²⁺ imaging methods. By applying simple Ca²⁺ indicators such as dextran or acetoxymethyl esters to more powerful genetically encoded Ca²⁺ indicators, zebrafish provides a transparent model where live Ca²⁺ imaging can be successfully achieved (Kettunen, 2012).

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381: Reduced, Release of BDNF (<https://aopwiki.org/events/381>)

Short Name: Reduced, Release of BDNF

AOPs Including This Key Event

AOP ID and Name	Event Type
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	KeyEvent

AOP ID and Name	Event Type
54: Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	KeyEvent
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 (https://aopwiki.org/aops/12)	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

BDNF plays a critical role in normal brain development in most vertebrates, primarily documented empirically in mammalian species. Klein et al. (2011) examined blood, serum, plasma and brain-tissue and measured BDNF levels in three different mammalian species: rat, pig, and mouse, using an ELISA method (Aid et al., 2007), whereas Trajkovska et al. 2007 determined BDNF levels in human blood.

There is compelling data that demonstrates the role of BDNF in brain development for many other taxa, including fish where it acts as neurotrophic factor in controlling cell proliferation (D'Angelo L (https://www.ncbi.nlm.nih.gov/pubmed/?term=D%27Angelo%20L%5BAuthor%5D&cauthor=true&cauthor_uid=23983038) et al., 2014; Heinrich and Pagtakhan, 2004) and birds where BDNF influences development of the brain area that involved in the song control (Brenowitz 2013) and the addition of new neurons to a cortical nucleus in adults. In the *Xenopus* visual system, BDNF acts as neurotrophic factor that mediates synaptic differentiation and maturation of the retinotectal circuit through cell autonomous TrkB signaling on retinal ganglion cells (Sanchez et al., 2006; Marshak et al., 2007).

How this Key Event Works

Biological state: BDNF belongs to a family of closely related neurotrophic factors named neurotrophins and is widely expressed in the developing and mature CNS. In the rodent cortex, postnatal BDNF expression is initially low but slowly increases to reach high levels around weaning. Therefore, BDNF expression peaks at a time when both structural and functional maturation of cortical circuitry occurs. During postnatal development, BDNF

levels are dynamically regulated, in part by neuronal activity dependent mechanisms (Waterhouse and Xu, 2009). Glutamate has been shown to increase the transcription and release of BDNF. Indeed, BDNF is synthesized, stored and released from glutamatergic neurons (Lessmann et al., 2003).

Biological compartments: BDNF initially is synthesized as precursor proteins (proBDNF), which is processed intracellularly to be transformed in its mature form (mBDNF) after proteolytically cleaved in the synaptic cleft by plasmin which is a protease activated by tissue plasminogen activator (tPA) (Cohen-Cory et al., 2010). proBDNF is constantly secreted while tPA release and mBDNF production depends on neuronal excitation (Head et al., 2009). Storage and activity-dependent release of BDNF has been demonstrated in both dendrites and axon terminals (Waterhouse and Xu, 2009). More specifically, in hippocampus, BDNF appears to be stored in dendritic processes of neurons (Balkowiec and Katz, 2002). BDNF is abundant in cerebellum and cortex and has also been measured in cerebrospinal fluid (CSF) (Zhang et al., 2008), whole blood, plasma, serum (plasma without clotting factors) and platelets (Trajkovska et al., 2007). BDNF has been found to be produced by astrocytes under both physiological and pathological conditions (Endo, 2005; Coco et al., 2013; Nelson and Alkon, 2014).

In humans, mBDNF is sequestered in platelets, consequently BDNF can reach all tissues and organs. Lymphocytic cells have been shown to express BDNF in vitro similarly to eosinophils, dendritic cells, and endothelial cells. The visceral and airway epithelium are also significant sources of BDNF. Female reproductive system including ovaries, placenta and uterus also express BDNF (Wessels et al., 2014).

General role in biology: The biological functions of mBDNF are mediated by binding to tyrosine kinase B (TrkB) receptor that leads to the activation of three major intracellular signalling pathways, including MAPK, PI3K and PLC γ 1 (Soulé et al., 2006). TrkB-mediated signaling regulates gene transcription in the nucleus through the activation of several transcription factors. These genes are involved in neurite outgrowth, synaptogenesis, synapse maturation and stabilization (Pang et al., 2004; Lu et al., 2005; Nelson and Alkon, 2014).

On the other hand, proBDNF binds to the p75 neurotrophin receptor (p75NTR) and activates RhoA, a small GTPase that regulates actin cytoskeleton polymerization leading to inhibition of axonal elongation, growth cone collapse, and apoptosis (Dubreuil et al., 2003; Yamauchi et al., 2004; Head et al., 2009).

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 BDNF protein and mRNA levels. Depending on the tissue or fluid measurements distinct methods are used.

Brain tissue: BDNF protein levels can be measured by commercial available antibody sandwich ELISA kits, Western blotting, immunohistochemistry and immunofluorescence. BDNF primers for different exons are available to determine mRNA levels by RT-PCR. The *Bdnf* gene consists of multiple alternative exons (ten in human, eight in rodents and six in lower vertebrates), and a single exon coding for the entire pro-BDNF protein (Cohen-Cory et al., 2010).

Cerebro-spinal fluid (CSF): There are available commercial antibody sandwich ELISA kits (Trajkovska et al., 2007) and immunobead-based multiplex assays for high throughput screening (Zhang et al., 2008).

Whole blood, serum, plasma and platelets: There are several commercial double antibody sandwich ELISA kits that can be used for identification of BDNF levels in biological fluids (Trajkovska et al., 2007).

Methodological considerations that have to be taken into account during sample preparation and measurement of BDNF by ELISA have been recently reviewed in Elfving et al. 2010. A study measuring BDNF by a commercially available ELISA kit in various tissues and biological liquids derived from distinct species revealed that BDNF is undetectable in mouse blood and pig plasma (Klein et al., 2011). This study also showed that in most cases BDNF levels are comparable to levels reported in humans and that there is positive correlation between blood BDNF levels and hippocampal BDNF levels in rats and pigs (Klein et al., 2011).

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AOP12

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55: N/A, Cell injury/death (<https://aopwiki.org/events/55>)

Short Name: N/A, Cell injury/death

AOPs Including This Key Event

AOP ID and Name	Event Type
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	KeyEvent
38: Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	KeyEvent
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 (https://aopwiki.org/aops/12)	KeyEvent
144: Lysosomal damage leading to liver inflammation (https://aopwiki.org/aops/144)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
human and other cells in culture	human and other cells in culture	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
Rattus norvegicus	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Cell death is an universal event occurring in cells of any species. [11]

How this Key Event Works

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 Ca^{2+} . 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 Ca^{2+} -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 cytochrome c in the case of Bax. tAIF translocates to cell nuclei, and together with cyclophilin A and phosphorylated histone H2AX (γ H2AX) is responsible for DNA cleavage, a feature of programmed necrosis. Activated calpain I has also been shown to cleave the plasma membrane Na^{+} - Ca^{2+} exchanger, which leads to build-up of intracellular Ca^{2+} , which is the source of additional increased intracellular Ca^{2+} . 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. 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 [1]. 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 [1][2]. Constitutively expressed nitric oxide synthase (nNOS) is a Ca^{2+} -dependent cytosolic enzyme that forms nitric oxide (NO) from L-arginine, and NO reacts with the free radical such as superoxide (O_2^-) to form the very toxic free radical peroxynitrite (ONOO^-). Free radicals such as ONOO^- , O_2^- and hydroxyl radical (OH^-) damage cellular membranes and intracellular proteins, enzymes and DNA [1], [2] [3][4].

How it is Measured or Detected

Necrosis:

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. [5]

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 including 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 [6].

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 [7].

Apoptosis:

AOP12

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 [8].

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. [9] [10]

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188: N/A, Neuroinflammation (<https://aopwiki.org/events/188>)

Short Name: N/A, Neuroinflammation

AOPs Including This Key Event

AOP ID and Name	Event Type
17: Binding to SH/selen-proteins can trigger neuroinflammation leading to neurodegeneration (https://aopwiki.org/aops/17)	KeyEvent
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 (https://aopwiki.org/aops/12)	KeyEvent
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent
3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent

Biological Organization

Level of Biological Organization

Tissue

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Neuroinflammation is observed in humans, monkey, rat, mouse and zebrafish associated to neurodegeneration or following toxicant exposure. Some references (not exhaustive list) are given below for illustration:

In humans: Venneti et al., 2006; In monkey (Macaca fascicularis): Charleston et al., 1994, 1996; In rat: Little et al., 2012; Eskes et al., 2002; In mouse: Liu et al., 2012; In zebrafish: Xu et al., 2014

How this Key Event Works

Neuroinflammation or brain inflammation differs from peripheral inflammation in that the vascular response and the role of peripheral bone marrow-derived cells are less conspicuous. The most easily detectable feature of neuroinflammation is the activation of microglial cells and astrocytes. It is evidenced by changes in shape, increased expression of certain antigens, and accumulation and proliferation of these glial cells in affected regions (Aschner, 1998; Graeber & Streit, 1990; Monnet-Tschudi et al, 2007; Streit et al, 1999; Kraft and Harry, 2011; Claycomb et al., 2013). Upon stimulation by cytokines or inflammasins (e.g. from pathogens or from damaged neurons), both glial cell types activate inflammatory signalling pathways, which result in increased expression and/or release of inflammatory mediators such as cytokines, eicosanoids, and metalloproteinases (Dong & Benveniste, 2001), as well as in the production of reactive oxygen (ROS) and nitrogen species (RNS) (Brown & Bal-Price, 2003). Different types of activation states are possible for microglia and astrocytes, resulting in pro-inflammatory or anti-inflammatory signalling and other cellular functions (such as phagocytosis) (Streit et al., 1999; Nakajima and Kohsaka, 2004). Therefore, neuroinflammation can have both neuroprotective/neuroreparative and neurodegenerative consequences (Carson et al., 2006 ; Monnet-Tschudi et al, 2007; Aguzzi et al., 2013 ; Glass et al., 2010). Under normal physiological conditions, microglial cells scan the nervous system for neural integrity (Nimmerjahn et al, 2005) and for invading pathogens (Aloisi, 2001; Kreutzberg, 1995; Kreutzberg, 1996; Rivest, 2009). They are the first type of cell activated (first line of defence), and can subsequently induce astrocyte activation (Falsig, 2008). Two distinct states of microglial activation have been described (Gordon, 2003; Kigerl et al, 2009; Maresz et al, 2008; Mosser & Edwards, 2008; Pergo et al; Ponomarev et al, 2005): The M1 state is classically triggered by interferon-gamma and/or other pro-inflammatory cytokines, and this state is characterized by increased expression of integrin alpha M (Itgam) and CD86, as well as the release of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6), and it is mostly associated with neurodegeneration. The M2 state is triggered by IL-4 and IL-13 (Maresz et al, 2008; Pergo et al, 2011; Ponomarev et al, 2007) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes. The activation of astrocytes by microglia-derived cytokines or TLR agonists resembles the microglial M1 state (Falsig 2006).

How it is Measured or Detected

Neuroinflammation, i.e. the activation of glial cells can be measured by quantification of cellular markers (most commonly), or of released mediators (less common). As multiple activation states exist for the two main cell types involved, it is necessary to measure several markers of neuroinflammation: 1. Microglial activation can be detected based on the increased numbers of labeled microglia per volume element of brain tissue (due to increase of binding sites, proliferation, and immigration of cells). A specific microglial marker, used across different species, is CD11b. Alternatively, various specific carbohydrate structures can be stained by lectins (e.g. IB4). Beyond that, various well-established antibodies are available to detect microglia in mouse tissue (F4/80), phagocytic microglia in rat tissue (ED1) or more generally microglia across species (Iba1). Transgenic mice are available with fluorescent proteins under the control of the CD11b promoter to easily quantify microglia without the need for specific stains. 1. The most frequently used astrocyte marker is GFAP (99% of all studies) (Eng et al., 2000). This protein is highly specific for astrocytes in the brain, and good clinically-validated

antibodies are available for immunocytochemical detection. In neuroinflammatory brain regions, the stain becomes more prominent, due to an upregulation of the protein, a shape change/proliferation of the cells, or better accessibility of the antibody. Various histological quantification approaches can be used. Occasionally, alternative astrocytic markers, such as vimentin of the S100beta protein have been used for staining of astrocytes (Struzynska et al., 2007). 2. All immunocytochemical methods can also be applied to cell culture models. 3. In patients, microglial accumulation can be monitored by PET imaging, using [¹¹C]-PK 11195 as microglial marker (Banati et al., 2002). 4. Activation of glial cells can be assessed in tissue or cell culture models also by quantification of sets of activation markers. This can for instance be done by PCR quantification of inflammatory factors, or by measurement of the respective mediators, e.g. by ELISA-related immuno-quantification. Such markers include: • Pro- and anti-inflammatory cytokine expression (IL-1 β ; TNF- α , IL-6, IL-4) ; or expression of immunostimulatory proteins (e.g. MHC-II) • Itgam, CD86 expression as markers of M1 microglial phenotype • Arg1, MRC1, as markers of M2 microglial phenotype (for description of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014)

Regulatory example using the KE:

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

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AOP12

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Adverse Outcomes

Title	Short name
N/A, Neurodegeneration (https://aopwiki.org/events/352)	N/A, Neurodegeneration
Impairment, Learning and memory (https://aopwiki.org/events/341)	Impairment, Learning and memory

352: N/A, Neurodegeneration (<https://aopwiki.org/events/352>)

Short Name: N/A, Neurodegeneration

AOPs Including This Key Event

AOP ID and Name	Event Type
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 (https://aopwiki.org/aops/12)	AdverseOutcome
17: Binding to SH/selen-proteins can trigger neuroinflammation leading to neurodegeneration (https://aopwiki.org/aops/17)	AdverseOutcome
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent

Biological Organization

Level of Biological Organization
Tissue

Evidence Supporting Applicability of this Event

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).

How this Key Event Works

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 including death of neurons. Neurodegeneration occurs in a large number of diseases that come under the umbrella of "neurodegenerative diseases" including, Huntington's, Alzheimer's and Parkinson's diseases. 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:

In the brain, the cerebral cortex is highly sensitive to heavy metal exposure. This may be due to differential accumulation, such as observed following high concentrations of mercury (Hamilton et al, 2011), or to differential vulnerability. The hippocampus is the most affected structure following exposure to trimethyltin (TMT) (Dey et al, 1997; Fiedorowicz et al, 2001; Fiedorowicz et al, 2008; Robertson et al, 1987), or mercury exposure during the developmental period (Falluel-Morel et al, 2012). Pb2+ affects the hippocampus and the frontal cortex (Schneider et al, 2012). In these sensitive regions, a decrease of synapses or cellular loss is observed (Corvino et al, 2013; Dey et al, 1997). Changes in genes involved in the amyloid cascade related to Alzheimer's disease were observed in the cortex of monkeys following Pb2+ exposure early in life (Zawia and Basha, 2005 ; Wu et al., 2008). In addition, aggregation of the amyloid peptide β was particularly enhanced in these monkeys after re-exposure to Pb2+ (Basha et al, 2005). These epigenetic modifications may be due to DNA methylation mediated in part through lead-induced dysregulation of methyltransferases (Schneider et al, 2013). The particular sensitivity of cortical areas to heavy metal exposure together with the increase of amyloid peptide deposition suggest a link between heavy metal exposure and Alzheimer's pathology (Castoldi et al, 2008; Mutter et al, 2004) . Paraquat and rotenone induce specific lesions in the substantia nigra (Costello et al, 2009; Wu et al, 2013), suggesting that these toxicants may be causally associated with Parkinson's disease.

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

- A build up of toxic proteins in the brain (Lansbury et al., 2006; Majd et al., 2015; Zaltieri et al., 2015)
- A loss of mitochondrial function that leads to the oxidative stress and creation of neurotoxic molecules that trigger cell death (apoptotic, necrotic or autophagy) (Lin and Beal, 2006; Braun , 2012; Betarbet et al., 2000; Zhu and Chu, 2010)
- Changes in the levels and activities of neurotrophic factors (Zuccato and Cattaneo, 2009; Michalski et al.,

2015)

- Variations in the activity of neural networks (Palop et al., 2006; Kann, 2015; Sala-Llonch et al., 2014).

Protein aggregation: the correlation between neurodegenerative disease and protein aggregation in the brain has long been recognized, but a causal relationship has not been unequivocally established (Lansbury et al., 2006). However, the causative link between mitochondrial dysfunction and its relationship to protein degradation and intracellular transport is well documented (Zaltieri et al., 2015). The dynamic nature of protein aggregation means that, despite progress towards understanding aggregation, 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, reduce the incidence of these disease (Gerard et al., 2010; McFarland and Okun, 2013).

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 neurodegenerative diseases there is strong evidence that mitochondrial dysfunction occurs early and acts causally in disease pathogenesis (Zaltieri et al., 2015). 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 hold some promise (Navneet et al., 2012).

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 disease, Alzheimer's disease and Parkinson's disease (Zuccato and Cattaneo, 2009; Michalski et al., 2015)). 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 neurodegenerative human diseases, with a view to ultimately influencing the clinical treatment of these conditions. Therefore BDNF treatment is often used as a beneficial and feasible therapeutic approach in the clinic setting (Bai et al., 2013; Nagahara and Tuszyński, 2011; Bradley 1999).

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 temporarily able to overcome. Variations in neuronal network activity are implicated in many brain diseases (Kann, 2015) as well as in aging (Sala-Llonch et al., 2014).

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 Examples Using This Adverse Outcome

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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341: Impairment, Learning and memory (<https://aopwiki.org/events/341>)

Short Name: Impairment, Learning and memory

AOPs Including This Key Event

AOP ID and Name	Event Type
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	AdverseOutcome
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	AdverseOutcome
54: Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	AdverseOutcome

AOP12

AOP ID and Name	Event Type
77: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1 (https://aopwiki.org/aops/77)	KeyEvent
78: Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1 (https://aopwiki.org/aops/78)	KeyEvent
87: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 2 (https://aopwiki.org/aops/87)	KeyEvent
88: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 3 (https://aopwiki.org/aops/88)	KeyEvent
89: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 4 (https://aopwiki.org/aops/89)	KeyEvent
90: Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2 (https://aopwiki.org/aops/90)	KeyEvent
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 (https://aopwiki.org/aops/12)	AdverseOutcome

Biological Organization

Level of Biological Organization
Individual

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
fruit fly	<i>Drosophila melanogaster</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7227)
zebrafish	<i>Danio rerio</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
gastropods	<i>Physa heterostropha</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=160004)

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Learning and memory have been studied in invertebrates such as gastropod molluscs and drosophila and vertebrates such as rodents and primates. Recently, larval zebrafish has also been suggested as a model for the study of learning and memory (Roberts et al., 2013).

How this Key Event Works

Learning can be defined as the process by which new information is acquired to establish knowledge by systematic study or by trial and error (Ono, 2009). Two types of learning are considered in neurobehavioral studies: a) associative learning and b) non-associative learning.

Associative learning is learning by making associations between different events. In associative learning, a subject learns the relationship among two different stimuli or between the stimulus and the subject's behaviour. Classical conditioning, operant conditioning and category learning are some examples of associative learning. On the other hand, non-associative learning can be defined as an alteration in the behavioral response that occurs over time in response to a single type of stimulus. Habituation and sensitization are some examples of non-associative learning. Another important type of learning is emotional learning and the simplest form of emotional regulation is extinction (Quirk and Mueller, 2008). During extinction, conditioned response to a stimulus decreases when the reinforcer is omitted and fear conditioning experiments help to elucidate the underlined mechanism.

The memory to be formed requires acquisition, retention and retrieval of information in the brain, which is characterised by the non-conscious recall of information (Ono, 2009). Memory is considered very important as it allows the subjects to access the past, to form experience and consequently to acquire skills for surviving purposes. There are three main categories of memory, including sensory memory, short-term or working memory (up to a few hours) and long-term memory (up to several days or even much longer). At the cellular level the storage of long-term memory is associated with increased gene expression and protein synthesis as well as formation of novel synaptic connections (Lynch, 2004).

Learning-related processes require neural networks to detect correlations between events in the environment and store these as changes in synaptic strength (Abbott and Nelson, 2000). Long-term potentiation (LTP) and long-term depression (LTD) are two fundamental processes involved in cognitive functions (Abbott and Nelson, 2000; Malenka and Bear, 2004), which respectively, strengthen synaptic inputs that are effective at depolarizing the postsynaptic neuron and weaken inputs that are not, thus reinforcing useful pathways in the brain. Synapses that are strengthened become more effective at depolarizing the postsynaptic neuron, eventually driving neuronal activity to saturation (Abbott and Nelson, 2000). As correlated activity of presynaptic and postsynaptic neurons drives strengthening of specific synapses, the postsynaptic neuron will be driven more strongly, and so presynaptic inputs that were initially only poorly correlated with postsynaptic firing will be better able to trigger firing of the postsynaptic neuron. This implies that nervous systems must have a matching set of plasticity mechanisms that counteract these destabilizing forces. The cortical and hippocampal pyramidal neurons have a target firing rate, and synaptic strengths are regulated to maintain these rates relatively constant in the face of perturbations in input channel (Burroone et al., 2002). This provides a robust mechanism for generating stability in network function in the face of learning-related changes in synaptic input. In principle, neurons could maintain stable firing rates through homeostatic regulation of many aspects of neuronal excitability. These possibilities include balancing inward and outward voltage-dependent conductances that determine firing properties generally called "intrinsic excitability" (Marder and Goaillard, 2006; Zhang and Linden 2003), regulating inhibitory and/or excitatory synaptic strength (Turrigiano, 2011) or synapse number (Kirov et al., 1999) or by adjusting the ease with which other forms of plasticity can be induced, so-called "metaplasticity" (Abraham and Bear, 1996). Evidence suggests that all of these mechanisms can contribute to the homeostatic regulation of neuronal firing rates in central circuits. Activity-dependent alteration in synaptic strength is a fundamental property of the vertebrate central nervous system and is thought to underlie learning and memory.

A major expression mechanism of synaptic scaling is changes in the accumulation of synaptic glutamate receptors. Central synapses typically cluster both AMPA receptors and NMDA receptors. AMPA receptors are ionotropic and carry out the majority of excitatory synaptic current in the central nervous system; NMDA receptors are also ionotropic but open as a function of voltage, flux calcium, and mediate a number of calcium-dependent forms of synaptic plasticity (Malenka and Bear, 2004). Synaptic scaling results in postsynaptic changes in both types of glutamate receptors (Stellwagen and Malenka, 2006; Watt et al., 2000) and can therefore be monitored by measuring changes in receptor accumulation at synapses.

The best characterized form of LTP occurs in the CA1 region of the hippocampus, in which LTP is initiated by transient activation of receptors and is expressed as a persistent increase in synaptic transmission through AMPA receptors followed by activation of NMDARs. This increase is due, at least in part, to a postsynaptic modification of AMPA-receptor function; this modification could be caused by an increase in the number of receptors, their open probability, their kinetics or their single-channel conductance. Summing up activity-dependent alteration in synaptic strength is a fundamental property of the vertebrate central nervous system that underlies learning and memory processes.

It is appropriate to state that while much emphasis has been given on the key role of the hippocampus in memory, it would probably be simplistic to attribute memory deficits solely to hippocampal damage (Barker and Warburton, 2011). There is substantial evidence that fundamental memory functions are not mediated by hippocampus alone but require a network that includes, in addition to the hippocampus, anterior thalamic nuclei,

mammillary bodies cortex, cerebellum and basal ganglia (Aggleton and Brown, 1999; Doya, 2000; Mitchell et al., 2002, Toscano and Guilarte, 2005). Each of these brain structures can be potentially damaged leading to more or less severe impairment of learning and memory.

Amnesia is defined as the impairment or loss of memory. Depending on the cause amnesia can be characterised as functional, organic amnesia or infantile amnesia. Dementia, is a brain disease that causes a long term and often gradual decrease in the ability to think and remember as well as problems with language, and a decrease in motivation (Solomon and Budson, 2011). It is an intellectual impairment observed mainly in elderly people due to the progress of a neurodegenerative disease. In younger people this type of impairment is known as presenile dementia. The most common affected areas include memory, visual-spatial, language, attention, and executive function (problem solving). Therefore, very often, short-time memory, mind, speech and motor skills are affected. Certain forms of dementia can be treated, to some extent. The most common form of dementia is Alzheimer's disease, which accounts for between 50 and 60 percent of all cases. Other types include vascular dementia and Lewy body dementia (Burns, 2009). Initial symptoms in Alzheimer's disease is memory impairment (for review, Arhavsky, 2010), in particular short-term/episodic memory, which depends largely on hippocampal system (for review, Storandt et al., 2009; Daulatzai, 2013). This pathological and age-related memory decline is believed to be a result of reduced synaptic plasticity, including changes in the NR2 subunit composition of the NMDA receptor (for review, Wang et al., 2014). It can then evolve towards a global loss of cognitive functions defined as dementia (for review, Larson et al., 1992).

In the past, the study of infant memory has relied in models and tests used in adults and more specific amnesic patients with hippocampal damage. For this reason, the infant memory has been distinguished to declarative or explicit memory and nondeclarative or implicit memory. However, in recent years this distinction such as explicit/implicit are no longer accepted especially in relation to hippocampal function as new theories have been emerged (reviewed in Mullally and Maguire, 2014). Furthermore, there are findings that even very young infants have a more adept and flexible memory system than was previously thought and neurobiological data derived from non-humans provide support to the new hypotheses about hippocampal development that would facilitate to interpret infant memory data from humans.

How it is Measured or Detected

In humans: The neuropsychological tests have been used for neurosensory assessment of humans including identification of altered neurobehaviours in vulnerable populations such as children (Rohlman et al., 2008). Intelligence tests, perceptual motor tests, planning tests, and logical, spatial, short term, long term, and working memory tasks can be used in neurobehavioral studies to assess learning and memory. The same test is also used to identify risks from occupational exposure to chemicals.

In laboratory animals: Current behavioural tests used for evaluating learning and memory processes in rats such as the *Morris water maze*, *Radial maze*, *Passive avoidance* and *Spontaneous alternation* are characterized in the KE *Decreased Neuronal Network Function*.

Cognitive function including learning and memory is an important endpoint required by the US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD 426). The methods applied to assess learning and memory have been reviewed (Markis et al., 2009) and discussed in the OECD Series on testing and assessment number 20, Guidance document for Neurotoxicity Testing (2004). This document is considered an essential supplement to a substantial number of already existing OECD Test Guidelines relevant for neurotoxicity testing.

Regulatory Examples Using This Adverse Outcome

Impairment of learning and memory is considered a chemically-induced adverse outcome that is used for risk assessment and management purposes. Neurotoxicity testing guidelines (OECD TG 424 and 426) are implemented on a number of occasions where the neurotoxic properties of a compound have to be assessed in order to comply with relevant EU regulations. These regulations are as follows: REACH regulation (EC, No 1907/2006), Plant protection products regulation (EC, No 1107/2009), Biocidal products regulation (EC, No 528/2012), Test methods regulation (EC, No 440/2008), Classification, labelling and packaging of substances and mixtures (EC, No 1272/2008) and Maximum residue levels of pesticides in or on food and feed of plant and animal origin regulation (EC, No 396/2005).

The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD 426) both require testing of learning and memory. These DNT Guidelines have been used to identify developmental neurotoxicity and adverse neurodevelopmental outcomes (Makris et al., 2009). Also in the frame of the OECD GD 43 (2008) on reproductive toxicity, learning and memory testing may have potential to be applied in the context of developmental neurotoxicity studies. However, many of the learning and memory tasks used in guideline studies may not readily detect subtle impairments in cognitive function associated with modest degrees of developmental thyroid disruption (Gilbert et al., 2012).

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Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding of antagonist, NMDA receptors	directly leads to	Inhibition, NMDARs	Strong	
Inhibition, NMDARs	directly leads to	Decreased, Calcium influx	Strong	
Decreased, Calcium influx	directly leads to	Reduced, Release of BDNF	Strong	
Reduced, Release of BDNF	directly leads to	N/A, Cell injury/death	Strong	
N/A, Cell injury/death	directly leads to	N/A, Neuroinflammation	Moderate	
N/A, Neuroinflammation	directly leads to	N/A, Neurodegeneration	Moderate	
N/A, Neurodegeneration	directly leads to	Impairment, Learning and memory	Strong	
N/A, Neurodegeneration	directly leads to	N/A, Neuroinflammation	Moderate	

Binding of antagonist, NMDA receptors leads to Inhibition, NMDARs
(<https://aopwiki.org/relationships/229>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	directly leads to	Strong	
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 (https://aopwiki.org/aops/12)	directly leads to	Strong	

Evidence Supporting Applicability of this Relationship

The biophysical properties of rat and human receptors have been mostly assessed through recombinant studies, whereas the pharmacological properties of rat and human NMDA receptors have not been fully explored and compared yet (Hedegaard et al., 2012). Mean channel open times for human NMDA receptor subtypes in

recombinant protein studies are similar to those of the corresponding rat NMDA receptor subtypes. However, mean single-channel conductances for human NMDA receptor subtypes appear lower than those of the corresponding rat NMDA receptor subtypes. Regarding pharmacological properties of the receptors, the differences were less than 2-fold and were not observed at the same subtypes for all the antagonists tested, suggesting that the molecular pharmacology of NMDA receptor is conserved between human and rat, although some inter-species differences are seen in IC₅₀ values using two-electrode voltage-clamp recordings (Hedegaard et al., 2012).

How Does This Key Event Relationship Work

It is well documented that prolonged/chronic antagonism of NMDARs triggers the downstream KE named inhibition of NMDARs. Shorter term binding to the same receptors may trigger different downstream KEs, such as up-regulation of the NMDARs, resulting in toxic increased influx of calcium and to cell death. Consequently, this information can be captured in other KERs and AOP.

Weight of Evidence

Biological Plausibility

There is structural mechanistic understanding supporting the relationship between MIE (NMDARs, binding of antagonists) and KE (NMDARs, inhibition). Crystal structure studies are used to study the binding of antagonists/agonists to NMDA receptors. In case of NMDAR antagonists, the binding to the receptor causes LBD conformation changes which promote channel closure leading to reduced Ca²⁺ influx (Blanke and VanDongen, 2009). This lack of measurable ion flux is applied as an indication of NMDAR inhibition.

Empirical Support for Linkage

Include consideration of temporal concordance here

In slices of cerebellum derived from postnatal days 6-30 (PND 6-30) Sprague Dawley rats, 10 µM MK-801 completely blocked evoked NMDA excitatory postsynaptic currents (EPSCs) as it has been demonstrated by patch clamp technique (Rumbaugh and Vicini, 1999). The same technique has been employed in cortical slices from C57BL/6 mice of both genders and different age groups (P8-12, P21-28 or P45-90), showing that 1 µM APV and 50 nM NVP-AAM077 antagonise a similar amount of NMDA receptor current independently of the age (de Marchena et al., 2008).

Pb²⁺ has potent inhibitory effects on the NMDA receptor (Alkondon et al., 1990; Guilarte and Miceli, 1992; Guilarte, 1997; Gavazzo et al., 2001). In rat hippocampal neurons, Pb²⁺ (2.5-50 µM) inhibits NMDA-induced whole-cell and single-channel currents in a concentration-dependent manner, suggesting that Pb²⁺ can decrease the frequency of NMDA-induced channel activation (Alkondon et al., 1990). In the same study, they have examined the effect of Pb²⁺ on the binding of [³H]MK-801 to the rat brain hippocampal membranes and showed that Pb²⁺ inhibits the binding of [³H]-MK-801 in a concentration dependent manner with an IC₅₀ value close to 7 µM (Alkondon et al., 1990). These inhibitory effects of Pb²⁺ on NMDA receptors activation appear to be age and brain region specific (Guilarte, 1997; Guilarte and Miceli, 1992). The Pb²⁺ IC₅₀ is significantly lower in cortical membranes prepared from neonatal than from adult rats, whereas the hippocampus is more sensitive than the cerebral cortex since the Pb²⁺ IC₅₀ is significantly lower in the hippocampus (Guilarte and Miceli, 1992). The number of [³H]-MK-801 binding sites associated with the high and low affinity sites of Pb²⁺ inhibition in the hippocampus of rats is increased as a function of age, peaking at PND 28 and 21 (Guilarte, 1997). High and low affinity Pb(2+)-sensitive [³H]-MK-801 binding sites have also been measured in the cerebral cortex during early development, but that has not been possible to evaluate after PND 14.

The developing brain is more sensitive than the adult brain to Pb²⁺-induced effects mediated through the NMDA receptor. Moreover, the hippocampus appears to be particularly vulnerable as in this brain structure NMDA receptors undergo subunit specific changes during developmental Pb²⁺ exposure (Guilarte and McGlothan, 1998). Exposure to Pb²⁺ during synaptogenesis causes decreased expression of hippocampal NR2A-subunit of NMDARs at synapses and increased targeting of NR2B-NMDARs to dendritic spines (without increased NR2B-NMDARs expression) (Nihei and Guilarte, 1999; Neal et al., 2011; Zhang et al., 2002).

Uncertainties or Inconsistencies

Pb²⁺ has been found to produce either potentiation or inhibition depending on: a) the subunit composition of NMDA receptors, b) endogenous glutamate concentration and c) Pb²⁺ dosage. In case that the NMDA receptors are saturated by agonist, Pb²⁺ at low concentrations (<1 µM) acts as a positive modulator of agonist action at NR1b-2AC and NR1a-2AB subunit complexes, whereas at higher concentrations, Pb²⁺ it behaves as a potent inhibitor of all recombinant NMDA receptors tested and was least potent at NR1b-2AC (Omelchenko et al., 1996; 1997), meaning that Pb²⁺ is not always acting as NMDAR inhibitor but it can also behave as NMDAR activator under certain conditions.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

To predict how potent an antagonist can be, the half maximal inhibition concentrations (IC₅₀) and the half maximal effective concentration (EC₅₀) of glutamate/glycine induced currents is measured in NMDA receptors from brain slices and cells or in recombinantly expressed receptors. Traynelis et al. 2010 summarised the IC₅₀

values for competitive, noncompetitive and uncompetitive antagonists in different subunits of NMDA receptors. The inhibitory effect (efficacy) of antagonists on NMDA receptors has been found to be dependent on:

- the type of subunits that form the NMDA receptors depending on the developmental stage
- the chemical structure of the antagonists
- the binding site of receptor that the antagonists prefer
- how tightly an antagonist binds to the receptor (affinity)

At CA3-CA1 synapses, NMDARs are largely composed of NR1 (NMDA receptor subunit 1)-NR2A or NR1-NR2B containing subunits. Recent, but controversial, evidence has correlated NR1-NR2A receptors with the induction of LTP and NR1-NR2B receptors with LTD. However, LTP can be induced by activation of either subtype of NMDAR and the ratio of NR2A:NR2B receptors has been proposed as an alternative determinant of the direction of synaptic plasticity (Mac Donald et al., 2006).

Pb2+: Although the NR2 subunits have different Zn²⁺ binding sites i.e. the NR2A-NMDAR binds Zn²⁺ at a high-affinity site (nM affinity) while the NR2B-NMDAR binds Zn²⁺ with lower affinity (μM range); the Pb²⁺ IC₅₀ for wild type NR2A-NMDARs was reported to be 1.3 μM, while the Pb²⁺ IC₅₀ of wild type NR2B-NMDARs was 1.2 μM (Gavazzo et al., 2008). Similar findings were published by Lasley and Gilbert (1999) using cortical neurons from adult rats. The IC₅₀ for Pb²⁺ ranged from 1.52 to 4.86 μM, with the ranking of Pb²⁺ potency in inhibition of NMDA receptor subunits to be NR1b-2A>NR1b-2C>NR1b-2D>NR1b-2AC after experiments that have been conducted in *Xenopus* oocytes injected with cRNAs for different combinations of NMDA receptor subunits (Omelchenko et al., 1997).

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Inhibition, NMDARs leads to Decreased, Calcium influx
(<https://aopwiki.org/relationships/213>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	directly leads to	Moderate	
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 (https://aopwiki.org/aops/12)	directly leads to	Strong	

Evidence Supporting Applicability of this Relationship

Besides the above studies described in rodents, intracellular Ca²⁺ regulation has been studied at the neuromuscular junction of larval Drosophila exposed to 0, 100 µM or 250 µM Pb²⁺ (He et al., 2009).

How Does This Key Event Relationship Work

The NMDA receptor is distinct in two ways: firstly, it is both ligand-gated and voltage-dependent and secondly, it requires co-activation by two ligands: glutamate and either D-serine or glycine.

NMDA receptor activation allows the influx of Ca²⁺ only when the receptor is occupied by L-glutamate or other agonists (and removal of Mg⁺⁺ block) resulting in the postsynaptic membrane depolarization. In contrast, binding of antagonist to NMDA receptor decreases or eliminates Ca²⁺ influx and consequently dramatically decreases intracellular influx of Ca²⁺ levels (reviewed in Higley and Sabatini, 2012).

Weight of Evidence

Biological Plausibility

The relationship between KE (NMDARs, Inhibition) and KE (Calcium influx, Decreased) is plausible as the function evaluation of NMDA receptors is commonly carried out by measurement of intracellular influx of Ca²⁺ upon NMDA receptor stimulation by agonist. Calcium imaging techniques have been extensively utilized to investigate the relationship between these two KEs. Almost 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 dendritic spine head reaching a concentration of ~10 µM (Higley and Sabatini, 2012). However, the majority of the ions are rapidly eliminated by binding Ca²⁺ proteins, reaching ~1 µM of free Ca²⁺ concentration (Higley and Sabatini, 2012).

In rat primary forebrain cultures, the intracellular Ca²⁺ increases after activation of the NMDA receptor and this increase 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).

Neurons in brain slices from wild-type (GluR ϵ 2+/+) mice showed increase of intracellular Ca²⁺ in the presence of 100 µM NMDA that was completely inhibited after exposure to 100 mM APV. In contrast, the NMDA-mediated increase in Ca²⁺ was absent in brain slices from GluR ϵ 2-/- mice that do not possess any functional NMDA receptors in the developing neocortex (Okada et al., 2003).

Empirical Support for Linkage

Include consideration of temporal concordance here

Pb²⁺: There are a few studies examining the effect of Pb²⁺ exposure on the changes in intracellular Ca²⁺. Incubation of rat synaptosomes with Pb²⁺ stimulates the activity of calmodulin reaching the higher effect at 30 µM, whereas higher concentrations of Pb²⁺ causes inhibition (Sandhir and Gill, 1994). Pb²⁺ exposure increases the activity of calmodulin by 45% in animal models. The IC50 values for inhibition of Ca²⁺ ATPase by Pb²⁺ has been found to be 13.34 and 16.69 µM in calmodulin-rich and calmodulin-depleted synaptic plasma membranes, respectively. Exposure of rats to Pb²⁺ has also inhibitory effect on Ca²⁺ ATPase activity, causing increase in

intrasyaptosomal Ca²⁺ (Sandhir and Gill, 1994). In embryonic rat hippocampal neurons, exposure to 100 nM Pb²⁺ for periods from 1 hour to 2 days shows decrease of intracellular Ca²⁺ by a calmodulin-dependent mechanism (Ferguson et al., 2000).

There is evidence that Pb²⁺ exposure affects Ca²⁺ homeostasis causing alterations in the phosphorylation state of different kinases. For example, Pb²⁺ has been shown to interfere with MAPK signaling as it increases the phosphorylation of both ERK1/2 and p38(MAPK) (Cordova et al., 2004). However, the findings regarding calcium/calmodulin kinase II (CamKII) activity are not clear (Toscano and Guilarte, 2005). On one hand, Pb²⁺ has been found to cause reduction of CREB phosphorylation in the hippocampus of rats exposed during brain development (Toscano et al., 2003; Toscano et al., 2002). On the other hand, the levels of phosphorylation of CamKII have not been explored but only the mRNA expression levels have been studied in rat pups on PND 25 that received Pb²⁺ (180 and 375-ppm lead acetate in food for 30 days) and reached blood Pb²⁺ levels 5.8 to 10.3 µg/dl on PND 55 (Schneider et al., 2012). More specifically, CamKIIα gene expression has been found to be very sensitive to Pb²⁺ exposure in the frontal cortex but not in the hippocampus, whereas CamKIIβ gene expression in both brain structures remained unchanged (Schneider et al., 2012).

Acute Pb²⁺ (10µM) exposure impairs LTP (125.8% reduction of baseline) in CA1 region of hippocampus derived from Sprague-Dawley rats (15-18 PND) as it has been recorded by whole cell patch-clamp technique (Li et al., 2006). In the same study, through calcium imaging, it has been shown in the 10mM caffeine-perfused cultured hippocampal neurons that 10µM Pb²⁺ reduces intracellular Fluo-4 fluorescence ratio to 0.44 (Li et al., 2006).

Pb²⁺ chronically or acutely applied, significantly reduces LTP in CA1 region of hippocampus from Wistar or Sprague-Dawley rats (30 and 60 PND) (Carpenter et al., 2002). These animals were exposed to Pb²⁺ via the mother's drinking water either through gestation and lactation (upto day 21) (perinatal), only by lactation through the mother's drinking water and then in the pup's drinking water (post) or from gestation (pre and post). The concentrations of Pb²⁺ used in the drinking water were 0.1 and 0.2%. In CA1, LTP has been reduced at both ages and Pb²⁺ concentrations or duration of exposure. In CA3, there have been no differences with time of exposure, but there was a dramatic difference in response as the age of animals increased. At 30 days LTP was significantly reduced, but at 60 days LTP was increased by about 30% (Carpenter et al., 2002). In the same brain structure and area (CA3) the effects of Pb²⁺ on LTP have been different in 30 PND and 60 PND rats after either acute perfusion of Pb²⁺ or from slices derived from rats after chronic developmental exposure to Pb²⁺, as inhibition of LTP has been recorded in 30 PND CA3, whereas potentiation has been measured in 60 PND CA3 with either exposure paradigm that have been attributed to possible involvement of protein kinase C (Hussain et al., 2000).

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Inhibition of NMDAR (KE up) (measurements, quantitative if available)	Reduced Ca 2+ influx (KE down) (measurements, quantitative if available)	References
Lead	CA1 pyramidal neurons derived from Sprague-Dawley rats (15-18 PND)	5-20 µM		25 min	By bath application of 5µM lead for 15 min prior to and 10 min after the tetanus, the LTP was induced to 151.4±3.5% of baseline. In the presence of 10µM lead, the LTP was reduced significantly to 125.8±2.9% of baseline. The reduction of 20µM lead to LTP was not significant with that of 10µM.	In the 10mM caffeine-perfused cultured hippocampal neurons, 10µM lead reduced intracellular Fluo-4 fluorescence ratio to 0.44±0.08	Li et al., 2006

		Rats	In CA1, LTP is exposed to reduced at both ages, lead via the and there were no mother's significant differences drinking in the effects of the water either two lead through concentrations or with gestation the duration of and exposure. In CA3 lactation (to there were no day 21) differences with time (perinatal), of exposure, but there only by was a dramatic lactation difference in response through the as a function of age. mother's At 30 days LTP was drinking significantly reduced, water and but at 60 days LTP then in the was increased by pup's about 30%. As in the drinking chronic exposure water until studies, lead reduced use (post) LTP in CA1 at both or from ages but reduced LTP gestation in CA3 in 30-day until use animals while (pre and potentiating LTP in post). 60-day animals.	Carpenter et al., 2002
Lead	CA1 region of hippocampus from Wistar or Sprague-Dawley rats (30 and 60 PND)	Two concentrations of lead were used in the drinking water 0.1 and 0.2%.	in utero and per os	

Uncertainties or Inconsistencies

The structural diversity of NMDA subunits can influence the functionality of the receptors and their permeability to Ca²⁺. For example, NR2B subunits show higher affinity for glutamate binding and higher Ca²⁺ permeability (reviewed in Higley and Sabatini, 2012). But NMDA receptor subunit composition is not the only parameter that influences Ca²⁺ entrance in the cytosol. Membrane potential due to pore blockade by extracellular Mg²⁺ and receptor phosphorylation are two additional regulator of Ca²⁺ influx through NMDA receptors (reviewed in Higley and Sabatini, 2012).

Entrance of Ca²⁺ into neuronal cell can also happen through KA and AMPA receptors but to a smaller extend compared to NMDA receptors (reviewed in Higley and Sabatini, 2012). However, recent findings suggest that AMPA receptors may also contribute to Ca²⁺ signalling during CNS development (reviewed in Cohen and Greenberg, 2008). Early in development cortical pyramidal neurons express calcium-permeable, GluR2 subunit-lacking AMPA receptors. During postnatal development these neurons undergo a switch in the subunit composition of AMPA receptors, expressing instead GluR2-containing, calcium-impermeable AMPA receptor suggesting that the main point entrance of Ca²⁺ at this developmental stage are NMDA receptors.

Furthermore, Ca²⁺ entry occurs through L- and H-type voltage-dependent Ca²⁺-channels (L-VDCCs) (Perez-Reyes and Schneider, 1994; Berridge, 1998; Felix, 2005) that are encountered in neurons, suggesting that there are more possible entrance sites for Ca²⁺ to get into the cytosol rather than only through NMDA receptors.

Interestingly, Pb²⁺ has the ability to mimic or even compete with Ca²⁺ in the CNS (Flora et al., 2006). Indeed, Pb²⁺ is accumulated in the same mitochondrial compartment as Ca²⁺ and it has been linked to disruptions in intracellular calcium metabolism (Bressler and Goldstein, 1991). So, it can be that the reduced levels of Ca²⁺ after Pb²⁺ exposure may not be attributed to NMDA receptor inhibition but also to the ability of this heavy metal to compete with Ca²⁺. To make things more complicated, recent findings suggest that BDNF can also acutely elicit an increase in intracellular Ca²⁺ concentration, which is attributed not only to the influx of extracellular Ca²⁺ but also to Ca²⁺ mobilization from intracellular calcium stores (Numakawa et al., 2002; He et al., 2005). These finding derive from primary cultures of cortical neurons (E18 or 2-3 PND), where BDNF-evoked Ca²⁺ signals have not been altered neither by tetrodotoxin nor by a cocktail of glutamate receptor blockers (CNQX and APV), pointing out the importance of BDNF in Ca²⁺ homeostasis (Numakawa et al., 2002; He et al., 2005).

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

No enough data is available to address the questions above.

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Decreased, Calcium influx leads to Reduced, Release of BDNF
(<https://aopwiki.org/relationships/347>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	indirectly leads to	Weak	
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 (https://aopwiki.org/aops/12)	directly leads to	Strong	

How Does This Key Event Relationship Work

Mainly, NMDA receptor activation initiates Ca²⁺-dependent signaling events that regulate the expression of genes involved in regulation of neuronal function including *bdnf* (reviewed in Cohen and Greenberg, 2008). Inhibition of NMDA receptors results in low levels of Ca²⁺ and decreased transcription of BDNF and consequently to low level of BDNF protein production and release.

Weight of Evidence

Biological Plausibility

BDNF transcription is induced by Ca²⁺ entering through either L type voltage gated calcium channel (L-VGCC) (Tao et al., 1998) or NMDA receptor (Tabuchi et al., 2000; Zheng et al., 2011) that can last up to 6 h. BDNF IV that is the most studied among its different exons has been shown to bind three Ca²⁺ elements within the regulatory region (reviewed in Zheng et al., 2012). One of these Ca²⁺ elements binds to CREB facilitating transcription. However, more transcription factors rather than only CREB are implicated in the transcription process of BDNF such as NFAT (nuclear factor of activated T cell), MEF2 (myocyte enhancer factor 2) and NF κ B (nuclear factor κ B) (reviewed in Zheng et al., 2012). The activation of the relevant transcription factor is triggered by the initial activation of CaM kinase, cAMP/PKA and Ras/ERK1/2 pathways mediated by the elevated intracellular Ca²⁺. Interestingly, inhibitory studies targeting different elements of these pathways report reduction at mRNA BDNF levels (reviewed in Zheng et al., 2012).

In particular, exon IV BDNF mRNA transcription is regulated by a transcriptional silencer, methyl-CpG binding protein 2 (MeCP2), demonstrating that epigenetic alterations can also regulate BDNF transcription. Increase of intracellular Ca²⁺ levels phosphorylates MeCP2, which inactivates its repressor function and permits the transcription of BDNF exon IV (Chen et al., 2003; Greer and Greenberg, 2008; Tao et al., 2009; Zhou et al., 2006). Indeed, NMDA receptor activation has been shown to upregulate BDNF transcripts containing exon IV not only via Ca²⁺-dependent CREB but also through Ca²⁺ activation of MeCP2 transcription (Metsis et al., 1993; Shieh et al., 1998; Tao et al., 1998; Tabuchi et al., 2000; Chen et al., 2003; Jiang et al., 2005; Zheng et al., 2011), whereas NMDAR antagonists decrease BDNF exon IV expression (Zafra et al., 1991; Stansfield et al., 2012). Furthermore, BDNF mRNA is also targeted in different locations within the cell during the process of translation, depending on the promoter used (reviewed in Tongiorgi et al., 2006).

Empirical Support for Linkage

Include consideration of temporal concordance here

There is no direct evidence linking reduced levels of Ca²⁺ to decreased BDNF levels as they have not been ever measured both in the same study after exposure to stressors. However, there are findings that strongly link the different elements of Ca²⁺-dependent signalling events to transcription of BDNF.

Pb²⁺: Pb²⁺ decreases the ratio of phosphorylated versus total MeCP2 and consequently MeCP2 maintains its repressor function and prevents BDNF exon IV transcription (Stansfield et al., 2012). MeCP2 gene expression in the frontal cortex is very sensitive to Pb²⁺ exposure while in the hippocampus, the same gene is affected only at the higher exposure group in rat pups with blood Pb²⁺ levels 5.8 to 10.3 μ g/dl on PND 55 (Schneider et al., 2012). In two different *in vivo* studies from the same research group, the use of doses of Pb²⁺ that result in learning and LTP deficits in rats causes decrease in phosphorylation of CREB in cerebral cortex at 14 PND and the same reduction in phosphorylation state of CREB in both cortex and hippocampus at PND 50 (Toscano et al., 2002; 2003). Interestingly, under similar experimental conditions no alteration at the phosphorylation state of CAMKII has been recorded (Toscano et al., 2005). In primary hippocampal neurons exposed to 1 μ M Pb²⁺ for 5 days during the period of synaptogenesis (DIV7–DIV12), both the cellular and extracellular proBDNF protein levels of mBDNF decrease with the latter to smaller extend (Neal et al., 2010). In the same *in vitro* model, Pb²⁺ also decreases dendritic proBDNF protein levels throughout the length of the dendrites and causes impairment of BDNF vesicle transport to sites of release in dendritic spines (Stansfield et al., 2012). Furthermore, Pb²⁺ treatment resulted in a specific reduction of *Bdnf* exon IV and IX mRNA transcripts causing no alteration in the expression of exons I and II (Stansfield et al., 2012). Rat pups on PND 25 exposed to Pb²⁺ (180 and 375-ppm lead acetate in food for 30 days) demonstrated blood Pb²⁺ levels 5.8 to 10.3 μ g/dl on PND 55 and show no

change at gene levels of BDNF (Schneider et al., 2012). In mouse embryonic stem cells (ESCs), *Bdnf* exon IV has been found to be down-regulated in cells treated with 0.1 μ M Pb, whereas *Bdnf* exon IX has been found up-regulated (Sánchez-Martín et al., 2013).

Uncertainties or Inconsistencies

In a gene expression study, where gene analysis has been performed in the hippocampus derived from male or female rats fed with 1500 ppm Pb²⁺-containing chow for 30 days beginning at weaning, two molecular networks have been identified that were different between male and female treated rats. In these networks, CREB was the highly connected node, common for both networks (Schneider et al., 2011). However, no change has been reported in the expression of *bdnf* gene neither in male nor in female rats treated with Pb²⁺ (Schneider et al., 2011).

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

No enough data is available to address the questions above.

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Reduced, Release of BDNF leads to N/A, Cell injury/death
(<https://aopwiki.org/relationships/353>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	indirectly leads to	Weak	
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 (https://aopwiki.org/aops/12)	directly leads to	Strong	

Evidence Supporting Applicability of this Relationship

The survival and antiapoptotic role of BDNF has been investigated not only in rodents but also in developing chicken neurons (Hallbook et al., 1995; Frade et al., 1997; Reinprecht et al., 1998). In invertebrates, only recently a protein with possible neurotrophic role has been identified but its influence and function in neuronal cell death of developing neurons has not been investigated yet (Zhu et al., 2008).

How Does This Key Event Relationship Work

BDNF influences the apoptosis occurring in developing neurons through two distinct mechanisms (Bernd, 2008). mBDNF can trigger prosurvival signaling after binding to TrkB receptor through inactivation of components of the cell death machinery and also through activation of the transcription factor cAMP-response element binding protein (CREB), which drives expression of the pro-survival gene Bcl-2 (West et al., 2001). On the other hand, proBDNF binds to the p75 neurotrophin receptor (p75NTR) and activates RhoA that regulates actin cytoskeleton polymerization resulting in apoptosis (Lee et al., 2001; Miller and Kaplan, 2001; Murray and Holmes, 2011). It is proved that reduced levels of BDNF can severely interfere with the survival of neurons in different brain regions, leading to cell death (Lee et al., 2001; Miller and Kaplan, 2001; Murray and Holmes, 2011).

Weight of Evidence

Biological Plausibility

BDNF mRNA levels dramatically increase between embryonic days 11 to 13 during rat development, playing important role in neuronal differentiation and survival (reviewed in Murray and Holmes, 2011). The latter has been supported by transgenic experiments where BDNF-/- mice demonstrated a dramatic increase in cell death among developing granule cells leading to impaired development of the layers of the cerebellar cortex (Schwartz et al., 1997). BDNF has also been shown to provide neuroprotection after hypoxic-ischemic brain injury in neonates (P7) but not in older (P21) animals (Cheng et al., 1997; Han and Holtzman, 2000). The neuroprotective role of BDNF has been further supported by the observed correlation between elevated BDNF protein levels and resistance to ischemic damage in hippocampus *in vivo* (Kokaia et al., 1996) and K⁺ rich medium-induced apoptosis *in vitro* (Kubo et al., 1995).

Empirical Support for Linkage

Include consideration of temporal concordance here

Several *in vitro* and *in vivo* studies on cortical neurons have demonstrated that the survival of developing

neurons is closely related with the activation of the NMDA receptors and subsequent BDNF synthesis/release that fully support the BDNF neurotrophic theory (Ikonomidou et al., 1999; Yoon et al., 2003; Hansen et al., 2004).

Pb2+: Neonatal mice exposed to Pb2+ (350 mg/kg lead twice every 4 h) and sacrificed after 8-24 h show increased apoptotic neurodegeneration above that seen in normal controls. This effect has been recorded only in animals treated with Pb2+ at PND 7, but not at PND 14 (Dribben et al., 2011), confirming the importance of the time of exposure during development in order for Pb2+ to induce apoptosis. Two to four weeks old rats treated for 7 days with 15 mg/kg daily dose of lead acetate show increased apoptosis in hippocampus (Sharifi et al., 2002). In rats (30 PND), it has also been shown that Pb2+ (2, 20 and 200 mg/kg/d) can induce apoptosis (Liu et al., 2010). However, in contrast to the first two *in vivo* studies, the animals in this experimental approach were old enough to evaluate the most sensitive window of vulnerability of developing neurons to Pb2+ exposure (Liu et al., 2010), confirming that only Pb2+ treatment during synaptogenesis can lead to neuronal cell apoptosis. *In vitro* evidence of apoptosis induced by Pb2+ also derive from PC12 cells exposed to Pb2+ (0.1, 1, 10 μ M) that have shown increased activation of caspase-3 (Xu et al., 2006). Besides PC12 cells (Xu et al., 2006; Sharifi and Mousavi, 2008), lead-induced apoptosis has also been studied in cultured rat cerebellar neurons (Oberto et al., 1996), hippocampal neurons (Niu et al., 2002) and retinal rod cells (He et al., 2000). No significant increase in LDH release was found in neuro-spheres derived from neural stem cells (NSCs) originating from E15 rat cortex (CX), striatum (ST) or ventral mesencephalon (VM) after assessing at 24 h intervals from day 1 through day 7 after addition of lead acetate (0.1–10 μ M) (Huang and Schneider, 2004). However, LDH release was increased 1 day after addition of 100 μ M lead acetate (1.6-2.1 fold depending on the brain region) and 3 days after addition of 50 μ M lead acetate to the culture medium (1.3-1.5 fold depending on the brain region). No significant cell loss was observed in cultures exposed to 0.1–10 μ M lead acetate for 7 days after staining with Hoechst 33342. In contrast, significant cell loss was observed 7 days after exposure to 50 μ M (35-50% depending on brain region) or 100 μ M lead acetate (60-75% depending on brain region) (Huang and Schneider, 2004). In primary rat hippocampal neurons exposed to 1 μ M Pb2+ for 5 days during the period of synaptogenesis (DIV7–DIV12), decreased cellular proBDNF protein (40% compared to control) and extracellular levels of mBDNF (25% compared to control) have been recorded (Neal et al., 2010). Significant reductions specifically in dendritic proBDNF protein levels throughout the length of the dendrites have also been described by Stansfield et al. (2012) after exposure to the same concentration of Pb2+ using this *in vitro* model. In an *in vivo* study, mice at PND 7 with mean Pb2+ blood levels of 8.10 μ g/mL have shown increased apoptosis in the cortex, hippocampus, caudate-putamen, and thalamus compared to controls with $F(1,14) = 19.5, 8.40, 4.15, 4.53$, respectively (Dribben et al., 2011). These Pb2+ levels in blood (Dribben et al., 2011) were a bit higher than the levels determined in Guilarte et al. 2003 (3.90 μ g/dl) that served as the base to calculate *in vitro* doses in Neal et al. 2010 and Stansfield et al. 2012.

Uncertainties or Inconsistencies

Pb2+: A number of studies demonstrate that deletion of BDNF does not lead to significant apoptotic cell death of neurons in the developing CNS (reviewed in Dekkers et al., 2013). In an *in vivo* Pb2+ exposure study, where female rats received 1,500 ppm prior, during breeding and lactation shows no changes at mRNA levels of BDNF in different hippocampus section derived from their pups (Guilarte et al., 2003). Regarding Pb2+, the pre- and neonatal exposure of rats to Pb2+ (Pb2+ blood levels below 10 μ g/dL) show a decreased number of hippocampus neurons but no morphological or molecular features of severe apoptosis or necrosis have been detected in tested brains (Baranowska-Bosiacka et al., 2013). In contrast to the lack of apoptotic signs, reduced levels of BDNF concentration (pg/mg protein) of BDNF in brain homogenates has been recorded in forebrain cortex (39%) and hippocampus (29%) (Baranowska-Bosiacka et al., 2013). Pregnant rats have been exposed to lead acetate (0.2% in the drinking water) after giving birth until PND 20. At PND 20, blood Pb2+ levels in pups reached at 80 μ g/dl. In these animals, the gene expression in different brain regions has been assessed and demonstrated that hippocampus is most sensitive with alterations beginning at PND 12 when caspase 3 mRNA increases after Pb2+ exposure (Chao et al., 2007). However, bcl-x and BDNF mRNA in the hippocampus have been significantly increased after caspase 3 increase, suggesting that the apoptotic signal activates a compensatory response by increasing survival factors like BDNF and that the temporality suggested in this AOP may not be accurate (Chao et al., 2007).

Some of the reported “inconsistencies” may be due to the lack of sufficient details in the reporting since publications vary in what they measure. Some of the referenced studies look at BDNF transcripts, others look at BDNF protein. BDNF processing is highly complex and different mRNA transcripts are known to be implicated in different cellular function.

Several studies addressing apoptosis mainly in the developing cerebral cortex have shown that more mechanism besides neurotrophic factors may be involved. Cytokines, as well as neurotransmitters can potentially activate a number of intracellular proteins that execute cell death (Henderson, 1996; Kroemer et al., 2009), meaning that further branches to this AOP might be added in the future.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

No enough data is available to address the questions above.

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N/A, Cell injury/death leads to N/A, Neuroinflammation
(<https://aopwiki.org/relationships/365>)

AOPs Referencing Relationship

AOP Name	Directness	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. (https://aopwiki.org/aops/48)	indirectly leads to	Weak	
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 (https://aopwiki.org/aops/12)	directly leads to	Moderate	

Evidence Supporting Applicability of this Relationship

California sea lions that have been exposed to the marine biotoxin DomA developed an acute or chronic toxicosis marked by seizures, whereas histopathological analysis revealed neuroinflammation characterised by gliosis (Kirkley et al., 2014).

How Does This Key Event Relationship Work

The pioneering work of Kreutzberg and coworkers (1995, 1996) has shown that neuronal injury leads to neuroinflammation, with microglia and astrocyte reactivities. Several chemokines and chemokines receptors (fraktalkine, CD200) control neuron-microglia interactions and a loss of this control can trigger microglial reactivity (Blank and Prinz, 2013; Chapman et al., 2000; Streit et al., 2001). Upon injury causing neuronal death (mainly necrotic), signals termed Damage-Associated Molecular Patterns (DAMPs) are released by damaged neurons and promote microglial reactivity (Marin-Teva et al., 2011; Katsumoto et al., 2014). Toll-like receptors (TLRs) are pattern-recognition receptors that recognize specific pathogen- and danger-associated molecular

signatures (PAMPs and DAMPs) and subsequently initiate inflammatory and immune responses. Microglial cells express Toll-like receptors (TLRs), mainly TLR-2, which can detect neuronal cell death (for review, see Hayward and Lee, 2014). TLR-2 functions as a master sentry receptor to detect neuronal death and tissue damage in many different neurological conditions including nerve trans-section injury, traumatic brain injury and hippocampal excitotoxicity (Hayward and Lee, 2014). Astrocytes, the other cellular actor of neuroinflammation (Ranshoff and Brown, 2012) are also able to sense tissue injury via TLR-3 (Farina et al., 2007; Rossi, 2015).

Weight of Evidence

Biological Plausibility

It is widely accepted that cell/neuronal injury and death lead to neuroinflammation (microglial and astrocyte reactivities) in adult brain. In the developing brain, neuroinflammation was observed after neurodegeneration induced by excitotoxic lesions (Acarin et al., 1997; Dommergues et al., 2003) or after ethanol exposure (Tiwari et al., 2012; Ahmad et al., 2016). It is important to note that physiological activation of microglial cells is observed during brain development for removal of apoptotic debris (Ashwell 1990, 1991). But exposure to toxicant (ethanol), excitotoxic insults (kainic acid) or traumatic brain injury during development can also induce apoptosis in hippocampus and cerebral cortex, as measured either by TUNEL, BID or caspase 3 upregulation associated to an inflammatory response, as evidenced by increased level of pro- inflammatory cytokines IL-1 β , TNF- α , of NO, of p65 NF- κ B or of the marker of astrogliosis, glial fibrillary acidic protein (GFAP), suggesting that, during brain development, neuroinflammation can also be triggered by apoptosis induced by several types of insult (Tiwari and Chopra, 2012; Baratz et al., 2015; Mesuret et al., 2014).

Empirical Support for Linkage

Include consideration of temporal concordance here

Pb

Neural stem cells (NSCs) derived from newborn were more sensitive to Pb treatment (0-200 microM for 48h) than NSCs derived from adult brain. Pb treatment induced a decrease in cell viability and an increase in the astrocytic marker GFAP, consistent with astrogliosis (Chan et al., 2013). Similar observations were made in 3D cultures prepared from fetal rat brain cells exposed to Pb (10-6 - 10-4 M for 10 days). Pb-induced neuronal death was evidenced by a decrease of cholinergic and GABAergic markers associated to a decrease in protein content, accompanied by microglial and astrocyte reactivities (Zurich et al., 2002). These effects were more pronounced in immature than in differentiated cultures (Zurich et al., 2002). In adult rats, exposure to 100 ppm of Pb during 8 weeks caused neuronal death, evidenced by an increase in apoptosis (TUNEL) that was associated to microglial reactivity and an increase in IL-1 β , TNF- α and i-NOS expression (Liu et al., 2012). Acute exposure to Pb (25 mg/kg, ip, for 3 days) increased GFAP and glutamate synthetase expression with impairment of glutamate uptake and probable neuronal injury (Struzunsk, 2000; Struzunsk et al., 2001).

Domoic acid

- Astrogliosis is one of the histopathological findings revealed by the assessment of brains derived from patients diagnosed with Amnesic Shellfish Poisoning (ASP) (reviewed in Pulido, 2008). In a reference study, where the brain of a patient after acute DomA intoxication has been examined in great detail gliosis has been detected in the overlying cortex, dorsal and ventral septal nuclei, the secondary olfactory areas and the nucleus accumbens (Cendes et al., 1995). Reactive astrogliosis has also been confirmed in the sixth cortical layer and subjacent white matter in the orbital and lateral basal areas, the first and second temporal gyri, the fusiform gyrus, the parietal parasagittal cortex, and the insula (Cendes et al., 1995).
- Adult rats have been assessed seven days after the administration of DomA (2.25 mg/kg i.p.) and revealed astrocytosis identified by glial fibrillary acidic protein (GFAP)-immunostaining and activation of microglia by GSI-B4 histochemistry (Appel et al., 1997). More investigators have suggested that DomA can activate microglia (Ananth et al., 2001; Chandrasekaran et al., 2004).
- DomA treatment (2 mg/kg once a day for 3 weeks) in mice significantly stimulates the expression of inflammatory mediators, including IL-1 β (1.7 fold increase), TNF- α (2 fold increase), GFAP (1.4 fold increase), Cox-2 (3 fold increase), and iNOS (1.6 fold increase) compared to controls (Lu et al., 2013).
- Adult female and male mice have been injected i.p. with 4mg/kg (LD50) of DomA and Real-time PCR has been performed in the brain derived at 30, 60 and 240 min post-injection. The inflammatory response element cyclooxygenase 2 (COX-2) has been found to be 8 fold increased at the 30 and 60 min time points and then showed a descent back toward basal expression levels by 240 min (Ryan et al., 2005).
- Adult male rats treated with 2 mg/kg DomA i.p. have been sacrificed after 3 or 7 d and shown that GFAP and lectin staining could identify regions of reactive gliosis within areas of neurodegeneration but at higher magnifications compared to the ones used for neurodegeneration (Appel et al., 1997; Scallet et al., 2005).
- At 5 days and 3 months following DomA administration of male Wistar rats, a large number of OX-42 positive microglial cells exhibiting intense immunoreactivity in CA1 and CA3 regions of the hippocampus have been detected. With an antibody against GFAP, immunoreactive astrocytes have been found to be sparsely distributed in the hippocampus derived from DomA treated rats after 3 months' time interval (Ananth et al., 2003). At 5 days after the administration of DomA, GFAP positive astrocytes have been found increased in the hippocampus (Ananth et al., 2003).

Uncertainties or Inconsistencies

Pb

Sobin and coworkers (2013) described a Pb-induced decrease in dentate gyrus volume associated with microglial reactivity at low dose of Pb (30 ppm), but not at high doses (330 ppm), plausibly due to the death of microglial cells at the high dose of Pb.

Pb decreased IL-6 secretion by isolated astrocytes (Quian et al., 2007). Such a decrease was also observed in isolated astrocytes treated with methylmercury, and was reverted in microglia astrocyte co-cultures, suggesting that cell-cell interactions can modify the response to a toxicant (Eskes et al., 2002). It is interesting to note that glial cells and in particular astrocytes are able to accumulate lead, suggesting that these cells may be also a primary target of lead neurotoxic effects (Zurich et al., 1998; Lindhal et al., 1999).

Domoic acid

Adult male and female Sprague Dawley rats have received a single intraperitoneal (i.p.) injection of DomA (0, 1.0, 1.8 mg/kg) and have been sacrificed 3 h after the treatment. Histopathological analysis of these animals has shown no alterations for GFAP immunostaining in the dorsal hippocampus and olfactory bulb, indicating absence of reactive gliosis (Baron et al., 2013).

The exposed zebrafish from the 36-week treatment with DomA showed no neuroinflammation in brain (Hiolski et al., 2014). At the same time, microarray analysis revealed no significant changes in *gfap* gene expression, a marker of neuroinflammation and astrocyte activation (Hiolski et al., 2014).

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Quantitative evaluation of this KER does not exist (gap of knowledge).

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N/A, Neuroinflammation leads to N/A, Neurodegeneration
(<https://aopwiki.org/relationships/208>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Binding to SH/selen-proteins can trigger neuroinflammation leading to neurodegeneration (https://aopwiki.org/aops/17)	directly leads to	Strong	Weak
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	directly leads to	Moderate	
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 (https://aopwiki.org/aops/12)	directly leads to	Moderate	

Evidence Supporting Applicability of this Relationship

The hypothesis of developmental origin of Pb-induced neurodegeneration was tested and observed in Zebra fish by Lee and Freeman (2014).

How Does This Key Event Relationship Work

It is well accepted that chronic neuroinflammation is involved in the pathogenesis of neurodegenerative diseases (McNaull et al., 2010; Tansey and Goldberg, 2010; Thundyil and Lim, 2015). Chronic neuroinflammation can cause secondary damage (Kraft and Harry, 2011). The mechanisms by which neuroinflammation (i.e. activated microglia and astrocytes) can kill neurons and induce/exacerbate the neurodegenerative process has been suggested to include the release of nitric oxide that causes inhibition of neuronal respiration, ROS and RNS production, and rapid glutamate release resulting in excitotoxic death of neurons (Brown & Bal-Price, 2003; Kraft & Harry, 2011; Taetsch & Block, 2013). Glial reactivity is also associated with an excessive production and release of pro-inflammatory cytokines that not only affect neurons, but also cause detrimental feedback effects on microglia (Heneka et al., 2014). For example, sustained exposure to bacterial lipopolysaccharide (LPS) or to other pro-inflammatory mediators was shown to restrict microglial phagocytosis of misfolded and aggregated proteins (Sheng et al., 2003). Systemic immune challenge during pregnancy leading to microglial activation caused increased deposition of amyloid plaques and tau hyperphosphorylation in aged mice (Krstic et al., 2012), suggesting that neuroinflammation is involved in the amyloid plaques and neurofibrillary tangles formation. There is further evidence that the formation of neurofibrillary tangles is caused by microglial cell-driven neuroinflammation, since LPS-induced systemic inflammation increased tau pathology (Kitazawa et al., 2005).

Weight of Evidence

Biological Plausibility

Neuroinflammation is a component of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Neumann, 2001; Mutter et al., 2004), playing a secondary or an active primary role in the disease process (Hirsch and Hunot, 2009). Mc Naull and coworkers (Mc Naull et al., 2010) suggested that early developmental onset of brain inflammation could be linked with late onset of Alzheimer's disease. A recent paper by Krstic and coworkers (2012) showed that a systemic immune challenge during late gestation predispose mice to develop Alzheimer's like pathology when aging, suggesting a causal link between systemic inflammation, neuroinflammation, and the onset of Alzheimer's disease. Regarding toxicant-induced neuroinflammation, microglial/astrocyte activation and chronic neuron damage may continue for years after initial exposure (Taetsch and Block, 2013), suggesting that chronic neuroinflammation and neurodegeneration have a slow and long term temporal evolution. Ongoing neuroinflammation can be visualized in patients using the positron emission tomography (PET) ligand [11C] (R)-PK11195 (Cagnin et al., 2001). Recent genome-wide association study (GWAS) analyses of sporadic Alzheimer's disease revealed a set of genes that point to a pathogenic role of neuroinflammation in Alzheimer's disease (for review, see Heneka et al., 2014). High levels of pro-inflammatory cytokines produced by activated microglia and astrocytes are detected in the brain of Alzheimer's subjects and animal models (Mc Geer and Mc Geer, 1998; Janelsins et al., 2005).

Empirical Support for Linkage

Include consideration of temporal concordance here

Pb

Rats treated from gestation day 5 till postnatal day 180 with a mixture of Pb/Cd/As showed in early adulthood increased levels of IL-1 β , IL-6 and TNF- α in hippocampus and frontal cortex associated with increased A β

levels, where Pb applied alone triggered maximal A β induction (Ashok et al., 2015). Similarly, monkeys exposed during infancy to Pb (from birth to 400 days to Pb 1 mg/kg/day) showed in aging (23 y old) an overexpression of APP and Abeta (Bihaci et al., 2011), and of Tau mRNA and protein (Bihaci and Zawa, 2013). Similar observations were made in old rats (18-20 months) when exposed to Pb (0.2% in drinking water) from postnatal day 1 to 20 (Basha et al., 2005; Zawia and Basha, 2005; Bihaci et al., 2014). This was associated with cognitive impairment, that was observed only if animals were exposed when young (Bihaci et al., 2014). Perinatal exposure to Pb leading to a blood concentration of 10 μ g/dl (a concentration considered as safe for human) promotes Tau phosphorylation in forebrain, cerebellum and hippocampus (Gassowska et al., 2016).

However, adult exposure may also increase the risk of neurodegeneration, as suggested by the two following studies:

- human Tg-SwD1 APP transgenic mice treated with Pb (27 mg/kg/day by gavage) for 6 weeks beginning at 8 weeks of age showed increased accumulation of Abeta and amyloid plaques (Gu et al., 2012).
- former organolead workers had increased tibia Pb level associated with persistent brain damage measured by MRI (Stewart et al., 2006).

Some in vitro and in vivo experiments show also that neuroinflammation can lead to degeneration:

- the conditioned medium of Pb-treated microglial cells (10 microM for 12h) caused the death of neuroblastoma cells (Kumawak et al., 2014).
- immature 3D cultures treated with Pb for 10 days exhibited neuroinflammation and neuronal death was exacerbated after an arrest of treatment of 10 more days, supporting the fact that neuroinflammation leads to neurodegeneration (Zurich et al., 2002).
- In vivo and in vitro experiments showed that Pb cause microglial activation, which upregulate the levels of pro-inflammatory cytokines (IL-1 β , TNF- α) and of iNOS and cause neuronal injury and neuronal death in hippocampus. These effects are significantly reversed by minocycline, an antibiotic blocking microglial reactivity, showing the essential role of neuroinflammation in hippocampal neurodegeneration (Liu et al., 2012)
- gestational exposure of mouse to Pb (0.1 mM in drinking water) led at PND 21 to increased brain mRNA expression of IL-6 and glial fibrillary acidic protein (GFAP) as marker of astrogliosis, as well as caspase 1 and NOS 2, suggesting a link between Pb-induced neuroinflammation and deleterious effects on neurons (Kasten-Jolly et al., 2011, 2012)

Domoic acid

DomA promotes the expression of inflammatory genes in the brain, such as cyclooxygenase 2 (COX2) and the development of neurodegeneration (Ryan et al., 2005). By using COX2 inhibitors that causes decrease the appearance of DomA-induced neurodegeneration, they have concluded that neuroinflammation contributes towards the development of neurodegeneration (Ryan et al., 2011).

Uncertainties or Inconsistencies

Long-term treatments with NSAIDs (non-steroidal anti-inflammatory drugs) have a preventive effect on Alzheimer's disease development (Pierzick and Behl, 2005), but such treatment has no effect or is even detrimental if applied once the disease is in an advanced stage (Lichtenstein et al., 2010). These ambivalent effects may be due to the dual role of neuroinflammation and to its complexity.

Serum Pb level negatively correlates with verbal memory score, but not with abnormal cognition in Alzheimer's disease (Park et al., 2014). Estimates of exposure are uncertain in epidemiological studies, because of the the long latency period between putative exposures during early life and late onset of Alzheimer's disease, even though bone Pb content is an accurate measure of historical Pb exposure in adults (Bakulski et al., 2012).

In addition to neuroinflammation or associated to neuroinflammation, other mechanisms may be involved in neurodegeneration with Abeta and tau accumulation: Pb-induced epigenetic modifications of genes involved in the amyloid cascade or tau expression may participate to the accumulation of Abeta and tau accumulation following developmental exposure to Pb (Zawia and Basha, 2005; Basha and Reddy, 2010). Also oxidative damage to DNA was shown to be involved in delayed effects observed in old rats (PD 600), if exposed early postnatally (PD 1 to 20) (Bolin et al., 2006).

Gap of knowledge: there are no studies showing that GLF-induced neuroinflammation leads to neurodegeneration.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

There are few studies where markers of neuroinflammation are measured simultaneously with markers of cell death and neurodegeneration. In addition, neuroinflammation is a complex KE, since the neurodegenerative consequences depend on the microglial phenotype, which is measured only in very recent studies. An attempt

to link KEup to KEdown quantitatively is provided below.

Endpoints relevant for KE _{up} Neuroinflammation	Endpoints relevant for KE _{down} Neurodegeneration	Model and treatments	Reference
IL-6, IL-1b, TNF-a increased about 2x in hippocampus and frontal cortex	Abeta 1-42 and Abeta 1-40 increased of 50% in frontal cortex and hippocampus Among individual metals, Pb triggered the maximum induction	Exposure to a mixture of arsenic (0.38 ppm), cadmium (0.098 ppm) and Pb (0.22 ppm) or Pb alone (2.2 ppm) Rat: from gestational day 05 to postnatal day 180. Observation in early adulthood	Ashok et al., 2015
Modulation of IL-6, TGF- β 1 and IL-1beta Upregulation of GFAP (astrocyte reactivity)	Caspase 1 and NOS2 gene expression increased	Mouse treated with Pb (0.1mM) in drinking water from gestation-day 8 to PND21	Kasten-Jolly et al., 2011, 2012
microglial reactivity about 3x, about 4X increase of IL-1 beta, TNF-alpha, iNOS Blockade by minocycline (in vivo and in vitro)	about 5x increase of neuronal death in hippocampus back to control levels in vivo and in vitro	rat exposed to Pb (100ppm) from 24 to 80 days of age hippocampal neurons+ microglia co-cultures (50 mmol /L Pb for 48 h)	Liu et al., 2012
microglial and astrocyte reactivities observed at the end of the 10-day treatment	Decrease in markers of cholinergic and GABAergic neurons that was exacerbated (30-60% increased) if harvest was performed not immediately after the 10-day treatment but after another 10-day period devoid of treatment	Immature 3D cultures of fetal rat brain cells Pb (10^{-6} - 10^{-4} M) applied for 10 days followed by another period of 10 days without treatment	Zurich et al., 2002

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N/A, Neurodegeneration leads to Impairment, Learning and memory
(<https://aopwiki.org/relationships/1069>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging (https://aopwiki.org/aops/12)	directly leads to	Strong	

How Does This Key Event Relationship Work

Animal models of neurodegenerative diseases, in particular Alzheimer's disease, contributed to the elucidation

of the link between amyloid protein and tau hyperphosphorylation and cognitive deficits. Bilateral injections of amyloid- β peptide in the frontal cortex of rats leads to progressive decline in memory and neurodegeneration in hippocampus (for review Eslamizade et al., 2016). Recent findings have shown that soluble forms of A β rather than insoluble forms (fibrils and plaques) are associated with memory impairment in early stages of Alzheimer's disease (for review Salgado-Puga and Pena-Ortega, 2015). Several lines of evidence suggest that the small oligomeric forms of A β and tau may act synergistically to promote synaptic dysfunction in Alzheimer's disease (for review Guerrero-Minóz et al., 2015). Some reports proposed the concept of imbalance between production and clearance of A β 42 and related A β peptides, as an initiating factor inducing hyperphosphorylation of tau and leading to neuritic dystrophy and synaptic dysfunction (for review Selkoe and Hardy, 2016). Recent trials of three different antibodies against amyloid peptides have suggested a slowing of cognitive decline in post hoc analyses of mild Alzheimer subjects (for review Selkoe and Hardy, 2016). Therefore cognitive deficits may be related to the level and extent of classical Alzheimer pathology landmarks, but it is also influenced by neurodegeneration (for review Braskie and Thompson, 2013). Indeed decrease in hippocampal volume due to widespread neurodegeneration and visualized by neuroimaging appeared to be a significant predictor of memory decline (for review Braskie and Thompson, 2016).

Weight of Evidence

Biological Plausibility

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

Empirical Support for Linkage

Include consideration of temporal concordance here

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

Uncertainties or Inconsistencies

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

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Endpoints relevant for KE _{up} Neurodegeneration in hippocampus and cortex	Endpoints relevant for KE _{down} Impairment of learning and memory	Model and treatments	Reference

0.5 -1x increase in amyloid peptides accumulation if early postnatal exposure	Decrease in cognitive functions (Morris water maze, Y maze testing for spatial memory and memory, a hippocampal formation-dependent task)	Mice exposed to Pb 0.2% in drinking water from PND 1 to 20 or from PND 1-20 and from 7-9 months of age	Bihaci et al., 2014
about 5x increase of neuronal death in hippocampus back to control levels in vivo and in vitro after minocycline treatment	Long Term Potentiation (LTP) was lost in Pb-treated rats and restores upon minocycline treatment	rat exposed to Pb (100 ppm) from 24 to 80 days of age	Liu et al., 2012
About 2x more Abeta ₁₋₄₀ and of Abeta ₁₋₄₂ in CSF, Cortex and hippocampus Twice more amyloid plaque load than control Pb co-localized with amyloid plaques.	Impaired spatial learning ability (Morris maze)	Human Tg-SWDI APP mice received by oral gavage 50mg/kg Pb once daily for 6 weeks. Pb level in brain 60 microg/dL (similar level than those found in children, Gu et al., 2011)	Gu et al., 2012

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N/A, Neurodegeneration leads to N/A, Neuroinflammation
(<https://aopwiki.org/relationships/207>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Binding to SH/selen-proteins can trigger neuroinflammation leading to neurodegeneration (https://aopwiki.org/aops/17)	directly leads to	Strong	Weak
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	directly leads to	Moderate	
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 (https://aopwiki.org/aops/12)	directly leads to	Moderate	

How Does This Key Event Relationship Work

According to its definition, neurodegeneration includes the death of neurons. Therefore, the KER describing the link between cell death and neuroinflammation is applicable to this KER. The fact that neuronal death can trigger neuroinflammation and that neuroinflammation can, in turn, cause neuronal degeneration, is known as a vicious circle, which is involved in the pathogenesis of neurodegenerative diseases (Griffin et al., 1998; McGeer and Mc Geer, 1998; Blasko et al., 2004; Cacquevel et al., 2004; Barbeito et al., 2010; Rubio-Perez and Morillas-Ruiz, 2012; Thundyil and Lim, 2015; Hayward and Lee, 2014). Proteinopathies associated with neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) may be sensed as damage associated molecular patterns (DAMPs) and thus activate microglia within the CNS. In animal neurodegeneration models and post-mortem brain samples from patients suffering from neurodegenerative disorders often revealed the presence of activated microglia and the accumulation of inflammatory mediators at the lesion sites, which suggests a continuous crosstalk between the brain immune system and the injured neurons during neurodegeneration. Microglia are typically activated acutely in response to an initial triggering insult, but their continued presence in large numbers around the lesion areas may actually promote neuronal death despite the absence of the initial triggering insult. Inflammatory factors being released by dying neurons and/or actively secreted from the activated microglia aid in maintaining the vicious cycle between activated microglia and damaged neurons (Thundyil and Lim 2015).

Weight of Evidence

Biological Plausibility

In Alzheimer's disease, Griffin and coworkers (1997) described the presence of reactive microglial cells inside the amyloid plaques and of reactive astrocytes around the plaques. Intra-cerebroventricular injections of beta-amyloid resulted in age-related increase in cholinergic loss and microglial activation (Nell et al., 2015). Increased neuronal expression of presequence protease (PreP) decreased the accumulation of beta-amyloid in synaptic mitochondria and the neuroinflammatory response (Fang et al., 2015), showing a link between the accumulation of insoluble proteins and neuroinflammation. In addition, having the apolipoprotein E4 (APOE) allele, the strongest genetic risk factor for the development of Alzheimer's disease, increases microglial reactivity in the amyloid plaques of a mouse model of beta-amyloid deposition, suggesting a role for APOE in modulation beta-amyloid-induced neuroinflammation in Alzheimer's disease progression (Rodriguez et al., 2014).

Empirical Support for Linkage

Include consideration of temporal concordance here

Binding of agonists to N-methyl-D-aspartate receptor (NMDAR) in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive function

DomA (0.75 mg/kg body weight) when administered intravenously in adult rats reveals neuronal degeneration

followed by glial activation (Ananth et al., 2001; 2003). More specifically, 5 days after DomA administration, Nissl staining of brain sections derived from DomA-treated animals have shown extensive neuronal damage in the pyramidal neurons of CA1, CA3 subfields and hilus of the dentate gyrus in the hippocampus. In the same brain areas, neuroinflammation has also been evident characterised by increased GFAP and OX-42 immunoreactivity at 5 days after DomA administration but not earlier (24 h) (Ananth et al., 2003). Previously, the same research team has shown increased number of stained degenerated neurons in the hippocampus by Nissl staining as early as 24 h following the administration of DomA, however, the degeneration has been found to be more severe after 5 days (Ananth et al., 2001).

Gap of knowledge: there are no studies showing that GLF-induced neurodegeneration leads to neuroinflammation.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Quantitative evaluation of these KERs, when KEup and KEdown are measured in the same experiment in a dose and time dependent manner following exposure to DomA or GLF is not available.

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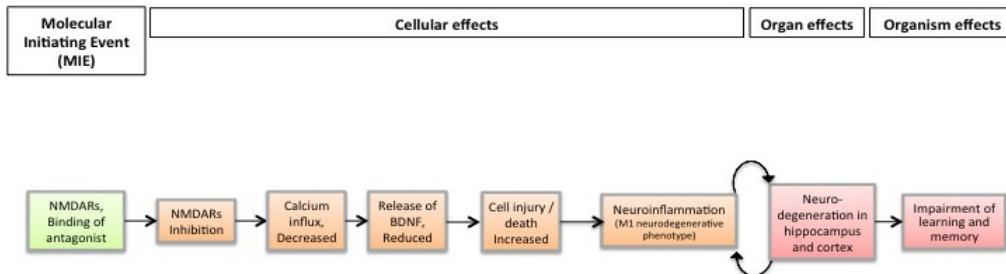
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Graphical Representation



Overall Assessment of the AOP

The aim of this AOP is to capture the KEs and KERs that occur after chronic binding of antagonist to NMDA receptors in neurons of hippocampus and cortex during brain development and that lead to neurodegeneration with impairment in learning and memory in later life. Neurodegeneration with accumulation of amyloid plaques and hyperphosphorylated tau, as well as cognitive deficit are associated to neurodegeneration of Alzheimer's type. Currently, the hypothesis of Landigan et al., (2005) of developmental origins of neurodegenerative diseases has been demonstrated in monkeys, in rats, mice and in zebra fish following Pb treatment (Zavia and Basha, 2005; Basha and Reddy, 2010; Bihaku et al., 2013; Bihaku et al., 2014 ; Lee and Freeman, 2014). There is strong agreement that Alzheimer's disease is progressive and that neurodegeneration is occurring mainly in hippocampus and cortex, associated with cognitive deficits (Schoemaker et al., 2014). This AOP uses the MIE and several KEs of the AOP 13 entitled "Binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities", with an additional KE: neuroinflammation and two AOs: an AO at the organ level: Neurodegeneration in hippocampus and cortex and an AO at the organism level: Impairment of learning and memory. Impairment of learning and memory is the same AO than in AOP 13, but the point is that this AO is detected when the brain is aging, and it is due to neurodegeneration with accumulation of amyloid peptides and tau hyperphosphorylation. Development Pb exposure has adverse effects on cognitive functioning that can persist into adulthood and may be exacerbated with aging (Schneider et al., 2013). Such delayed effects may be due to epigenetic effects of developmental Pb exposure on DNA methylation mediated at least in part through dysregulation of methyltransferases observed often at the lowest level of exposure (Schneider et al., 2013). The fact that neuroinflammation triggered during early brain development was shown to cause Alzheimer pathology when aging (Krstic et al., 2012), suggests that chronic neuroinflammation may play a causal role in cognitive decline in aging. A recent report described a mechanistic link between chronic inflammation and aging microglia; and a causal role of aging microglia in neurodegenerative cognitive deficits: A sirtuin 1 (SIRT1) deficiency was observed in aging microglia, leading to a selective activation of IL1- β transcription mediated through hypomethylation of IL-1 β proximal promoter exacerbating aging or tau-associated cognitive deficits (Cho et al. 2015). Taken together, these data suggest that Pb-induced neuroinflammation during brain development may underlie the delayed effects on cognitive deficits in aging, as depicted in the proposed AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
During brain development	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Monkey	Monkey	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
zebrafish	Danio rerio	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

This AOP is not sex dependent. Regarding the life stage applicability, MIE induced during brain development can have consequences when brain is aging, according to the hypothesis proposed by Landrigan and coworkers (2005). However, it is also possible that the AO does not depend exclusively on developmental exposure, since cumulative occupational exposure also decreased cognitive functions in aging (Stewart et al., 2006).

Essentiality of the Key Events

Table: Essentiality of KEs

	Defining Question	High (Strong)	Moderate	Low (Weak)
2 Support for Essentiality of KEs	Are downstream KEs and/or the AO prevented if an upstream KE is blocked ?	Direct evidence from specifically designed experimental studies illustrating essentiality for an expected modulating factor at least one of the important KEs (e.g. augments a KE leading to stop/reversibility studies, to increase in KE down antagonism, knock out or AO models, etc.)	Indirect evidence that sufficient modification of at least one of the factor attenuates or stops the increase in KE downstream antagonism, knock out or AO models, etc.)	No or contradictory experimental evidence of the essentiality of any of the KEs
KE1 NMDARs inhibition	STRONG	Activation of NMDAR results in LTP, which is related to increase synaptic strength and memory formation in hippocampus (Johnston et al., 2009).		
KE2 Calcium influx decreased	STRONG	In CNS, many intracellular responses to modified calcium level are mediated by calcium/calmoduline-regulated protein kinases (Wayman et al., 2008). Mice with a mutation of calmoduline kinase II, which is abundantly found in hippocampus, have shown spatial learning impairment (Silva et al., 1992)		
KE3 Release of BDNF, reduced	STRONG	BDNF serves essential function in synaptic plasticity (Poo, 2001) and is crucial for learning and memory processes (Lu et al., 2008). Precursor form of BDNF and mature BDNF are decreased in the preclinical stages of Alzheimer's disease (Peng et al., 2005)		
KE4 Cell Injury/death, increased	STRONG	Several studies dealing with postnatal administration of NMDAR antagonists such as MK 801, ketamine or ethanol have shown a devastating cell apoptotic degeneration in several brain areas of animal models resulting in learning deficits (Creeley and Olney, 2013)		

KE5 Neuroinflammation	MODERATE	<p>Rationale: Rats treated with Pb from PND 24 to 80 showed a neuroinflammatory response associated with neuronal death in hippocampus and LTP impairment. These effects were significantly reversed by administration of minocycline, an antibiotic known to block microglial reactivity (Liu et al., 2012), demonstrating the essentiality of neuroinflammation for neurodegeneration in hippocampus and impairment of memory processes. In addition, the fact that neuroinflammation triggered during brain development by a systemic immune challenge caused Alzheimer's like pathology (Krstic et al., 2012), showed the central role of neuroinflammation in this pathology. In addition, in a mouse model of Alzheimer's disease, the blockade of microglial cell proliferation and the shifting of the microglial inflammatory profile to an anti-inflammatory phenotype by inhibiting the colony-stimulating factor 1 receptor on microglial cells, prevented synaptic degeneration and improved cognitive functions (Olmos-Alonso et al., 2016). This latter experiment has not been done during brain development. But the hypothesis is that a chronic neuroinflammation during a prolonged period increased the risk to develop an Alzheimer's neurodegenerative disease in aging (Krstic and Knuesel, 2013).</p> <p>However, as other mechanisms such epigenetic modifications can lead to accumulation of amyloid plaques- and tau hyperphosphorylation-related neurodegeneration, and due to some inconsistencies of anti-inflammatory treatments as protection against the neurodegenerative process, the essentiality of Neuroinflammation was considered as moderate.</p>
AO (at organ level) Neurodegeneration in hippocampus and cortex	STRONG	<p>Several studies described Pb-induced accumulation of amyloid peptides and hyperphosphorylated and Pb-induced cell injury/death in hippocampus or decrease in hippocampal volume, what are all well accepted landmarks of Alzheimer's pathology (Lloret et al., 2015). As described in AOP 48, neurodegeneration can lead to "Decreased neuronal network function" which in turn leads to "impairment of learning and memory", which is also considered as a hallmark of Alzheimer's pathology (Schoemaker et al., 2014).</p> <p>However, there is some controversy about the relationship between increased accumulation of amyloid plaques and increased cognitive deficits: Lichtenstein and coworkers (2010) described that accumulation of amyloid plaques reaches a plateau, whereas a temporal relationship is observed between increased microglial activation, widespread degeneration (decreased hippocampal volume) and increased cognitive deficits. Therefore the essentiality for accumulation of amyloid and tau to cognitive deficits should be considered as moderate. But, as cell injury/death in hippocampus and cortex or decrease in hippocampal volume due to widespread neurodegeneration is strongly associated to impairment in learning and memory, the essentiality of this KE has been rated as strong.</p>
AO (at organism level) Impairment of learning and memory	STRONG	<p>Neurodegenerative diseases are complex and multifactorial, depend on gene-environment interactions, and have a slow temporal evolution (Sherer et al., 2002; Steece-Collier et al., 2002; Tsang and Soong, 2003); Mutter et al., 2004). Although a direct association of Pb with Alzheimer's development is not supported by epidemiological studies, a cohort study reported that bone Pb levels were associated with poor cognitive performance scores in old workers, suggesting that past Pb exposure can contribute to late cognitive deterioration (Dorsey et al., 2006). But despite the few epidemiological studies, the evidence is more solid in experimental studies (for review, see Chin-Chan et al., 2015). Cumulative organo-lead-exposed workers developed persistent brain lesions and progressive decline in cognitive functions (Stewart et al., 2006); and in a subcohort of the Veterans Affairs Normative aging study, it was found that Pb was associated with an increase in plasma homocysteine, a risk factor for cardiovascular diseases and neurodegeneration (Bakulski et al., 2014). Therefore, long term exposure to environmental toxicants such as Pb during brain development or exposure later in life can be considered as a risk factor for the development of neurodegenerative diseases in aging.</p>

Weight of Evidence Summary

1. Concordance of dose-response and temporal concordance between KEs and the AO

AOP12

It is difficult to analyze the dose-response relationships between the different KEs, because of the long temporal delay between MIE and AO ; because no study has analyzed them simultaneously, and because of the difficulties in extrapolating in vitro to in vivo data. As the apical KEs and AO occur and can be measured years after exposure, even when Pb blood level has returned to normal, measurement of bone Pb content has been proposed as a measurement of historical Pb exposure in adults (Bakulski et al., 2012, 2014). The following table gives an overview of the doses/concentrations and exposure duration at which the different KEs were measured.

KE1	KE2	KE3	KE4	K5	AO at organ level	AO at organism level
NMDAR inhibition	Calcium influx, decreased	BDNF release, decreased	Cell injury/death	Neuroinflammation	Neurodegeneration with amyloid plaques and tau hyperphosphorylation	Impairment of learning and memory

2. Strength, consistency and association of AO and MIE

The accepted molecular mechanism of action of the chemical initiator Pb is inhibition of NMDARs (Alkondon et al., 1990; Gavazzo et al., 2001, 2008; Guilarte et al., 1992; Omelchenko et al., 1997) and several experimental studies in rat, monkey and zebra fish linked chronic exposure to Pb during brain development to Alzheimer's like neurodegeneration with cognitive deficits (Zawia and Basha, 2005; Basha and Reddy, 2010; Bihaqui et al., 2013; Bihaqui et al., 2014 ; Lee and Freeman, 2014). However, although other inhibitors of NMDARs exist, none of them except Pb has been studied for long term effects and for linking the MIE to this AO. This is certainly a weakness of this AOP to have a single chemical initiator for the empirical support.

3. Biological Plausibility, and empirical support

	Defining Question	High /Strong	Moderate	Low/weak
Support for Biological Plausibility of KERs	Is there a mechanistic (i.e. structural or functional) relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance	The KER is plausible based on analogy to accept biological relationship but scientific understanding is not completely established	There is empirical support for a statistical association between KEs but the structural or functional relationship between them is not understood
MIE to KE inhibition of NMDARs		Extensive understanding Limited conflicting data		
KE NMDAR inhibition to KE calcium influx, decreased		Extensive understanding Limited conflicting data		
KE calcium influx, decreased to KE release of BDNF, decreased		Extensive understanding Limited conflicting data		
KE release of BDNF, decreased to KE Cell Injury/death		Extensive understanding Limited conflicting data		

KE Cell injury/death to KE Neuroinflammation			<p>The general mechanisms linking cell injury/death to neuroinflammation is well accepted. However, it is mainly described in adult brain. However, a neuroinflammatory response was found following Pb exposure of 3D cultures during synaptogenesis and myelination (Zurich et al., 2002). A controversy exists about apoptosis and neuroinflammation, but some empirical evidences have been provided.</p> <p>The fact that cell injury/death leads to neuroinflammation and that neuroinflammation leads to neurodegeneration is known as vicious circle and is involved in neurodegenerative diseases, suggesting that neuroinflammation exacerbates the neurodegenerative process (Griffin et al., 1998; 2006)</p>	
KE Neuroinflammation to AO Neurodegeneration in Hippocampus and cortex	<p>In adult, the early involvement of neuroinflammation in the neurodegenerative process is widely accepted.</p> <p>In immature brain, one study in mice link gestational induction of neuroinflammation to late neurodegeneration with accumulation of aberrant amyloid and tau (Kristic et al., 2012).</p>		<p>There are <i>in vitro</i> experimental evidences following Pb exposure linking neuroinflammation to extensive neuronal death in immature cells.</p> <p><i>In vivo</i>, There are several studies linking early Pb exposure to late neurodegeneration in several species.</p> <p>However, the mechanisms involved is epigenetic modifications of genes involved in the amyloid cascade. Such epigenetic modifications may be due to ROS released by the neuroinflammatory process (Bolin et al., 2006).</p> <p>Therefore the link may be indirect and needs further analyses.</p>	

AO Neurodegeneration in hippocampus and cortex to AO		The role of hippocampus in memory processes is well accepted. Alterations of LTP in hippocampus of rats exposed to Pb has been described (Liu et al., 2012), as well as preferential accumulation of hyperphosphorylated tau in frontal cortex of mice exposed during development to Pb. These mice exhibited cognitive deficit when aging (Bihaci et al., 2014).		

Quantitative Consideration

With an Adverse Outcome occurring after such a long delay after the MIE, it is extremely difficult to make a quantitative link, since the AO can occur when serum Pb levels have returned to normal. Bakulski and coworkers (2012) therefore proposed measuring Pb bone content as an index of historical Pb exposure. Regarding the KER "cell injury/death to neuroinflammation", it is accepted that neuronal injury may be sufficient to trigger a neuroinflammatory response. But, because of the neuroprotective or neuroreparative potential of neuroinflammation, it is possible that the consequences of neuroinflammation will be in a first step positive, with microglia expressing the M2 phenotype. After an exposure arrest and a temporal delay (Sandström et al., 2014), or in presence of cell death (Nakajima and Kohsaka, 2004; Hanish and Kettenmann, 2007), microglia can acquire the M1 neurodegenerative phenotype. Therefore, it is rather the qualitative phenotype of neuroinflammation that will induce neurodegeneration. However, a possible correlation of increased microglial reactivity, measured by PET and a decrease in hippocampal volume, measured by MRI, suggests, in advanced Alzheimer's disease, a possible link between the intensity of neuroinflammation and the neurodegenerative consequences (Lichtenstein et al., 2010).

Considerations for Potential Applications of the AOP (optional)

This AOP aims at giving a conceptual framework to mechanistically understand apical hazard, which can occur long after initial exposure; an hazard, which is not tested for in the standard neurotoxicity testing.

The KE "neuroinflammation", which is shared with other AOPs, appears to play an early and central role in the neurodegenerative process (Eikelenboom et al., 2000; Whitton, 2007; Krstic et al., 2012). Neuroinflammation is observed in most neurodegenerative diseases including Alzheimer's disease (Whitton, 2007 ; Tansey and Goldberg, 2009 ; Nirjanjan, 2014 ; Verhratiky et al., 2014). Neuroinflammation can also be triggered by several classes of toxicants (Monnet-Tschudi et al., 2007). Any toxicant able to trigger a neuroinflammatory response expressing the neurodegenerative M1 phenotype should be considered as a risk factor for neurodegenerative diseases. Therefore, testing for toxicant-induced neuroinflammation should be used as an endpoint in regulatory toxicology. The standard neurotoxicity testing does not require to measure any marker of neuroinflammation, except for fuel additives, where testing for a potential increase in glial fibrillary acidic protein (GFAP), as marker of astrocyte reactivity, is mandatory according to US EPA (40 CFR 79.67).

The evolution of regulation towards mechanistically-driven approaches for supporting hazard identification implies also the development of in vitro testing. Three-dimensional cultures, prepared from fetal rat brain cells, exhibiting an histotypic organization comprising all types of brain cells (specifically microglial cells and astrocytes, as effector cells of neuroinflammation) and allowing long time maintenance for repeated exposure and for studying the evolution of neuroinflammatory phenotypes are already available (Alépée et al., 2014; Monnet-Tschudi et al., 2007 ; Sandström et al., 2014). Similar 3D cultures prepared from human pluripotent stem cells are in development (Schwartz et al., 2016; Stoppini et al., in press).

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