

OECD Series on Adverse Outcome Pathways No. 6

Adverse Outcome Pathway on binding of agonists to ionotropic glutamate receptors in adult brain leading to excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment

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OECD

Foreword

This Adverse Outcome Pathway (AOP) on Binding of agonists to ionotropic glutamate receptors in adult brain leading to excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Group of the National Coordinators for the Test Guidelines Programme (WNT). The AOP has been reviewed internally by the EAGMST, externally by experts nominated by the WNT, and has been endorsed by the WNT and the Task Force on hazard Assessment (TFHA) in April 2016.

Through endorsement of this AOP, the WNT and the TFHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to declassification of this AOP on 17 June 2016.

This document is being published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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ADVERSE OUTCOME PATHWAY ON BINDING OF AGONISTS TO IONOTROPIC GLUTAMATE RECEPTORS IN ADULT BRAIN LEADING TO EXCITOTOXICITY THAT MEDIATES NEURONAL CELL DEATH, CONTRIBUTING TO LEARNING AND MEMORY IMPAIRMENT.

Short name: ionotropic glutamatergic receptors and cognition

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Abstract

Under physiological conditions activation of glutamate ionotropic receptors such as N-methyl-D-aspartate (NMDARs), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPARs) and kainate (KARs) is responsible for basal excitatory synaptic transmission and main forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) that are fundamental for learning and memory processes (Schrattenholz and Soskic, 2006). However, sustained (direct or indirect) over-activation of these receptors can induce excitotoxic neuronal cell death. Indeed, mainly increased Ca^{2+} influx through NMDARs promotes many pathways of toxicity due to generation of free radical species, reduced ATP production, endoplasmic reticulum (ER) stress and protein aggregation. Neuronal injury induced by over-activation of these receptors and the excessive Ca^{2+} influx is considered an early key event of excitotoxicity. Additionally, the excessive activation of NMDARs has been found to play a significant role in a variety of neurological disorders ranging from acute hypoxic-ischemic brain injury to chronic neurodegenerative diseases (Mehta et al., 2013). The proposed AOP is relevant to adult neurotoxicity testing. A molecular initiating event (MIE) has been defined as a direct binding of agonists to NMDARs or indirect, through prior activation of AMPARs and/or KARs resulting in sustained NMDARs over-activation causing excitotoxic neuronal cell death, mainly in hippocampus and cortex, two brain structures fundamental for learning and memory processes. The AOP is based on the empirical support describing (1) domoic acid (DomA) induced excitotoxicity triggered by indirect (through KARs/AMPARs) NMDARs over-activation leading to impaired learning and memory and (2) glufosinate (GLF) induced excitotoxicity that through direct binding to NMDARs causes convulsions and memory loss. GLF is the methylphosphine analog of L-glutamate, used as a component of bactericidal and fungicidal herbicidal. DomA, a natural toxin that accumulates in mussels and shellfish is also an analogue of L-glutamate and among the most prominent features described after human exposure to DomA is memory impairment (Lefebvre and Robertson, 2010). DomA and GLF are described as the examples of the stressors due to large amounts of published data (especially in the case of DomA), however this AOP is relevant to any agonist that directly or indirectly cause NMDARs over-activation. Some of the known agonists selective for the NMDARs are derived from the naturally occurring amino acids such as ibotenic acid, homocysteine and l-aspartate and polyamines like spermidine.

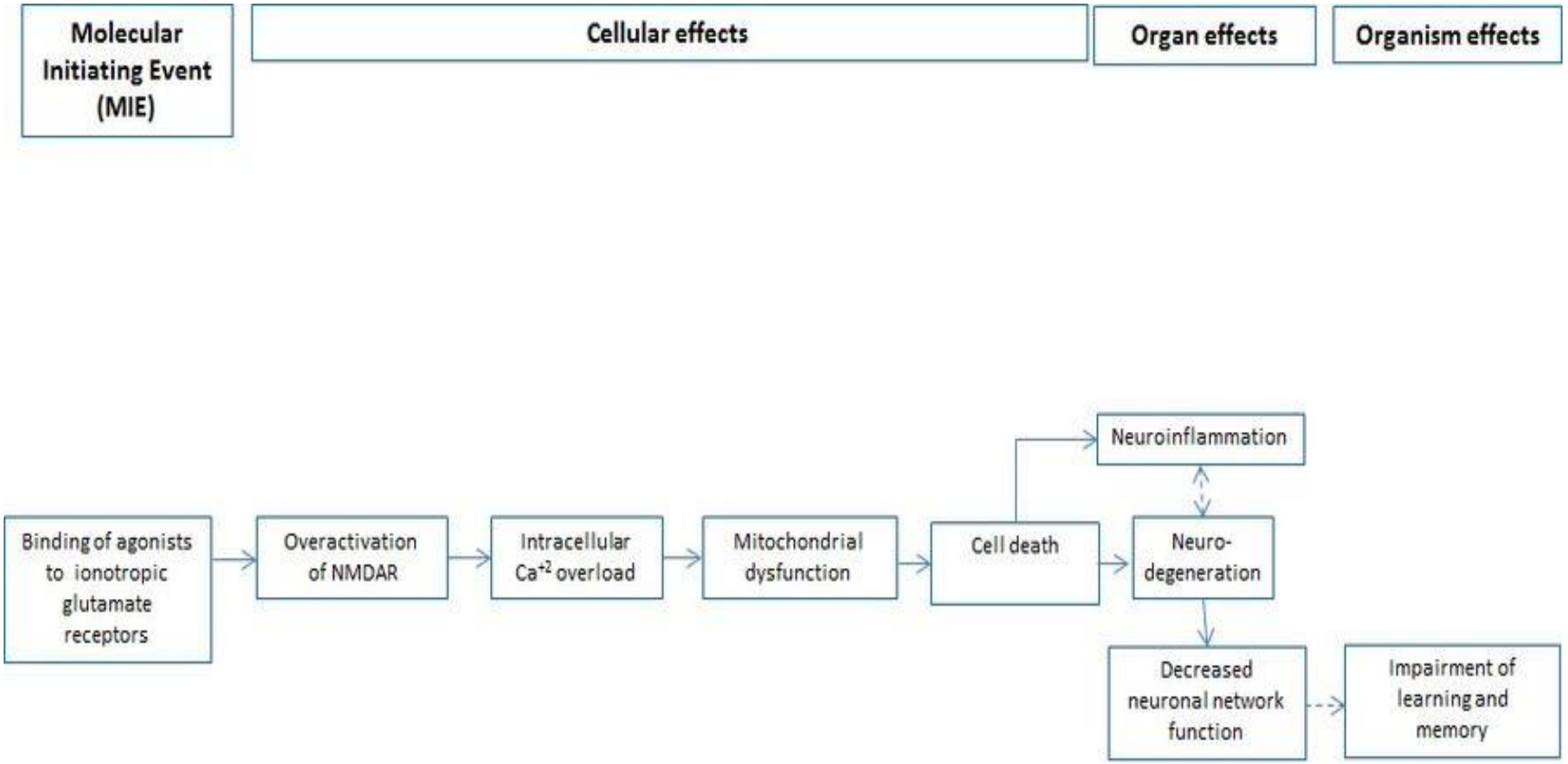
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Summary of the AOP: Graphical Representation of AOP



Key events

Molecular Initiating Event

Molecular Initiating Event
Inotropic glutamate receptors, Binding of agonist

Ionotropic glutamate receptors, Binding of agonist

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	MIE	Strong

Chemical Initiators

The following are chemical initiators that operate directly through this Event:

1. Domoic acid

How this Key Event works

Level of biological organisation
Molecular

The MIE of this AOP can be triggered by direct binding of an agonist to NMDARs or indirectly through initial activation of KA/AMPARs. Indeed, binding of agonist to KA/AMPARs results in ion influx (Na^+ and a small efflux of K^+) and glutamate release from excitatory synaptic vesicles causing depolarisation of the postsynaptic neuron (Dingledine et al., 1999). Upon this depolarisation the Mg^{2+} block is removed from the pore of NMDARs, allowing sodium, potassium, and importantly, calcium ions to enter into a cell. At positive potentials NMDARs then show maximal permeability (i.e., large outward currents can be observed under these circumstances). Due to the time needed for the Mg^{2+} removal, NMDARs activate more slowly, having a peak conductance long after the KA/AMPAR peak conductance takes place. It is important to note that NMDARs conduct currents only when Mg^{2+} block is relieved, glutamate is bound, and the postsynaptic neuron is depolarized. For this reason the NMDA receptors act as "coincidence detectors" and play a fundamental role in the establishment of Hebbian synaptic plasticity which is considered the physiological correlate of associative learning (Daoudal and Debanne, 2003; Glanzman, 2005). Post-synaptic membrane depolarisation happens almost always through activation of KA/AMPARs (Luscher and Malenka, 2012). Therefore, a MIE of this AOP is defined as binding of an agonist to these three types of ionotropic receptors (KA/AMPA and

NMDA) that can result in a prolonged overactivation of NMDARs through (a) direct binding of an agonist or (b) indirect, mediated through initial KA/AMPARs activation. The excitotoxic neuronal cell death, triggered by sustained NMDARs overactivation in the hippocampus and/or cortex leads to the impaired learning and memory, defined as the adverse outcome (AO) of this AOP.

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, stabilisation, 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, stabilisation 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 characterised by higher affinity for Glu, slower activation and desensitisation kinetics, higher permeability for calcium (Ca^{2+}) and susceptibility to potential-dependent blockage by magnesium ions (Mg^{2+}). 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 Mg^{2+} and Zn^{2+} ions, allowing the flow of Na^{+} and Ca^{2+} ions into the cell and K^{+} out of the cell which is voltage-dependent. Ca^{2+} 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

1. Ex vivo: The most common assay used is the NMDA receptor (MK801 site) radioligand competition binding assay (Reynolds and Palmer, 1991; Subramaniam and McGonigle, 1991; http://pdsp.med.unc.edu/UNC-CH%20Protocol%20Book.pdf; 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). The saturation binding of radioligands are used to measure the affinity (Kd) and density (Bmax) of kainate and AMPA receptors in striatum, cortex and hippocampus (Kürschner et al., 1998).

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 (Neville and Lytton, 1999; Mazumder and Borah, 2014).

Evidence Supporting Taxonomic Applicability

Name	Scientific Name	Evidence	Links
Drosophila melanogaster	Drosophila melanogaster	Strong	NCBI
Rattus norvegicus	Rattus norvegicus	Strong	NCBI
Primates sp. BOLD:AAA0001	Primates sp. BOLD:AAA0001	Strong	NCBI
human	Homo sapiens	Strong	NCBI
mice	Mus sp.	Strong	NCBI

The major determinants for ligand e.g. for both co-agonist glycine binding and L-glutamate binding are well conserved between species from lower organism to mammals (reviewed in Xia and Chiang, 2009). PCR analysis, cloning and subsequent sequencing of the seal lion NMDA receptors showed 80% homology to those from rats, but more than 95% homologus to those from dogs (Gill et al., 2010).

Evidence for Chemical Initiation of this Molecular Initiating Event

L-Glutamate and glycine (or D-serine) are endogenous agonists that bind to the LBD of specific NMDA receptor subunits. Here listed some known agonists for NMDA receptor, some of them are specific to the NR1 subunit and some others to the NR2 subunit (reviewed in Traynelis et al., 2010).

Specific to NR1
Glycine, l-Serine, d-Serine, l-Alanine, d-Alanine, d-Cycloserine, HA 966, (+)-(1-hydroxy-3-aminopyrrolidine-2-one,) β-Cl-d-Alanine, β-F-dl-Alanine, tri-F-dl-Alanine, ACPC, 1-aminocyclopropane-1-carboxylic acid, ACBC, 1-aminocyclobutane-1-carboxylic acid, GLYX-13.

Specific to NR2
l-Glutamate, d-Glutamate, l-Aspartate, d-Aspartate, N-Methyl-l-aspartate, N-Methyl-d-aspartate, SYM208,1 l-Homocysteinsulfinate, d-Homocysteinsulfinate, l-Homocysteate, d-Homocysteate, l-Cysteinesulfinate, l-Cysteate, d-Cysteate, Homoquinolinate, Ibotenate, (R,S)-(Tetrazol-5-yl)glycine, L-CCG-IV, (2S,3R,4S)-2-(carboxycyclopropyl)glycine, trans-ACBD, trans-1-aminocyclobutane-1,3-dicarboxylate, cis-ADA, cis-azetidine-2,4-dicarboxylic acid, trans-ADC, azetidine-2,4-dicarboxylic acid, cis-ACPD, (1R,3R)-aminocyclopentane-cis-dicarboxylate, cis-2,3-Piperidinedicarboxylic acid, (R)-NHP4G, 2-(N-hydroxylpyrazol-4-yl)glycine, (R,S)-Ethyl-NHP5G, 2-(N-hydroxypyrazol-5-yl)glycine, (R)-Propyl-NHP5G, 2-(N-hydroxypyrazol-5-yl)glycine.

Domoic acid (DomA) is structurally similar to kainic acid (KA) and both of them are analogues of the excitatory neurotransmitter L-glutamate. DomA induces excitotoxicity by an integrative action on ionotropic glutamate receptors at pre- and post-synaptic sides. DomA directly activates KA/AMPARs receptors followed by indirect activation of the NMDARs. Indeed, indirect activation of NMDARs by DomA is linked to the fact that KA and AMPA receptors activated by DomA induce increased levels of intracellular Ca^{2+} and Na^{+} which, in turn, causes endogenous glutamate release that subsequently potentiates activation of NMDARs (Berman and Murray, 1997; Berman et al., 2002; Watanabe et al., 2011). DomA has been demonstrated through both in vitro and in vivo approaches to indirectly activate the NMDARs (reviewed in Pulido, 2008).

Glufosinate (GLF) ((RS)-2-amino-4-(hydroxy(methyl)phosphonoyl)butanoic acid, phosphinothricin) is a phosphorus containing amino acid herbicide that is naturally occurring as a component of the bacteria-derived bactericidal and fungicidal tripeptides bialaphos and phosalacine (Lanz et al., 2014). There are studies suggesting that convulsive and amnesic effects of GLF are mediated through direct binding and activation of NMDAR (Lantz et al., 2014; Matsumura et al., 2001). GLF agonist action at the NMDAR is expected to occur through direct interaction with the glutamate binding site and requires binding of the glycine co-agonist as well as release of the magnesium block from the channel pore.

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Key events

Key Event
NMDARs, Overactivation
Calcium influx, Increased
Mitochondrial dysfunction, N/A
Cell death, N/A
Neurodegeneration, N/A
Neuroinflammation, N/A
Neuronal network function in adult brain, Decreased

1. NMDARs, Overactivation

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KE	Moderate

How this Key Event works

Level of biological organisation
Molecular

Biological state: Please see MIE NMDARs, Binding of antagonist
Biological compartments: Please see MIE NMDARs, Binding of antagonist
General role in biology: Please see MIE NMDARs, Binding of antagonist
The above chapters belong to the AOP entitled: *Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities* since the general characteristic of the NMDA receptor biology is the same for both AOPs.
Additional text, specific for this AOP:
At resting membrane potentials, NMDA receptors are inactive. Depending on the specific impulse train received, the NMDA receptor activation triggers long term potentiation (LTP) or long-term depression (LTD) (Malenka and Bear, 2004; Luscher and Malenka, 2012). LTP (the opposing process to LTD) is the long-lasting increase of synaptic strength. For LTP induction both pre- and postsynaptic neurons need to be active at the same time because the postsynaptic neuron must be

depolarized when glutamate is released from the presynaptic bouton to fully relieve the Mg^{2+} block of NMDARs that prevents ion flows through it. Sustained activation of AMPA or KA receptors by, for instance, a train of impulses arriving at a pre-synaptic terminal, depolarises the post-synaptic cell, releasing Mg^{2+} inhibition and thus allowing NMDA receptor activation. Unlike GluA2-containing AMPA receptors, NMDA receptors are permeable to calcium ions as well as being permeable to other ions. Thus NMDA receptor activation leads to a calcium influx into the post-synaptic cells, a signal that is instrumental in the activation of a number of signalling cascades (*Calcium-dependent processes are describe in Key Event Calcium influx, increased*). Postsynaptic Ca^{2+} signals of different amplitudes and durations are able to induce either LPT or LTD.

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

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

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

How it is Measured or Detected

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 Ca^{2+} by the patch clamp technique. This method relies on lack of measurable ion flux when NMDA ion channel is closed, whereas constant channel specific conductance is recorded at the open state of the receptor (Blanke and VanDongen, 2009). Furthermore, this method is based on the prediction that activation or inhibition of an ion channel results from an increase in the probability of being in the open or closed state, respectively (Ogdon and Stanfield, 2009; Zhao et al., 2009).

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

Microelectrode array (MEA) recordings are used to measure mainly spontaneous network activity of cultured neurons (Keefer et al., 2001, Gramowski et al., 2000 and Gopal, 2003; Johnstone et al., 2010). However, using specific agonists and antagonists of a receptor, including NMDAR, MEA technology can be used to measure evoked activity, including glutamatergic receptor function (Lantz et al., 2014). For example it has been shown that MEA-coupled neuronal cortical networks are very sensitive to pharmacological manipulation of the excitatory ionotropic glutamatergic transmission (Frega et al., 2012). MEAs can also be applied in higher throughput platforms to facilitate screening of numerous chemical compounds (McConnell et al., 2012).
Excessive excitability can be also measured directly by evaluating the level of the extracellular glutamate using the enzyme-based microelectrode arrays. This technology is capable of detecting glutamate in vivo, to assess the effectiveness of hyperexcitability modulators on glutamate release in brain slices. Using glutamate oxidase coated ceramic MEAs coupled with constant voltage amperometry, it is possible to measure resting glutamate levels and synaptic overflow of glutamate after K(+) stimulation in brain slices (Quintero et al., 2011).
Neuronal network function can be also measured using optical detection of neuronal spikes both in vivo and in vitro (Wilt et al., 2013).

Drebrin immunocytochemistry: drebrin, a major actin-filament-binding protein localized in mature dendritic spines is a target of calpain mediated proteolysis under excitotoxic conditions induced by the overactivation of NMDARs. In cultured rodent neurons, degradation of drebrin was confirmed by the detection of proteolytic fragments, as well as a reduction in the amount of full-length drebrin. The NMDA-induced degradation of drebrin in mature neurons occurres concomitantly with a loss of f-actin. Biochemical analyses using purified drebrin and calpain revealed that calpain degraded drebrin directly in vitro. These findings suggest that calpain-mediated degradation of drebrin is mediated by excitotoxicity, regardless of whether they are acute or chronic. Drebrin (A and E) regulates the synaptic clustering of NMDARs. Therefore, degradation of drebrin can be used as a readout for excitotoxicity induced by NMDAR overactivation. Degradation of drebrin can be evaluated quantitatively by Western blot analysis (mRNA evel) or by immunocytochemistry (at protein level) (Chimura et al., 2015: Sekino et al., 2006).

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

Evidence Supporting Taxonomic Applicability

Name	Scientific Name	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus sp.	Strong	NCBI
rat	Rattus sp.	Strong	NCBI

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

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2. Intracellular Calcium overload, Increased

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KE	Moderate

How this Key Event works

Level of biological organisation
Cellular

For the relevant paragraphs below please see AOP entitled *Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities.*
Biological state: KE Calcium influx, Decreased
Biological compartments: KE Calcium influx, Decreased
General role in biology: KE Calcium influx, Decreased

The text specific for this AOP:
It is now well accepted that modest activation of NMDARs leading to modest increases in postsynaptic calcium are optimal for triggering LTD (Lledo et al. 1998; Bloodgood and Sabatin, 2007; Bloodgood et al. 2009), whereas much stronger activation of NMDARs leading to much larger increases in postsynaptic calcium are required to trigger LTP (Luscher and Malenka, 2012; Malenka 1994). Indeed, high-frequency stimulation causes a strong temporal summation of the excitatory postsynaptic potentials (EPSPs), and depolarisation of the postsynaptic cell is sufficient to relieve the Mg^{2+} block of the NMDAR and allow a large amount of calcium to enter into the postsynaptic cells. Therefore, intra-cellular calcium is measured as a readout for evaluation NMDAR stimulation.

How it is Measured or Detected

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

Evidence Supporting Taxonomic Applicability

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

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3 Mitochondrial dysfunction, N/A

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KE	Strong
Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KE	
Inhibition of Complex I of the mitochondrial respiration chain leading to neurodegeneration.	KE	Strong
Lysosomal damage leading to liver inflammation	KE	Strong

How this Key Event works

Level of biological organisation
Cellular

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

Mitochondria can be found in all cells and are considered the most important cellular consumers of oxygen. Furthermore, mitochondria possess numerous redox enzymes capable of transferring single electrons to oxygen, generating the superoxide (O_2^-). Some mitochondrial enzymes that are involved in reactive oxygen species (ROS) generation include the electron-transport chain (ETC) complexes I, II and III; pyruvate dehydrogenase (PDH) and glycerol-3-phosphate dehydrogenase (GPDH). The transfer of electrons to oxygen, generating superoxide, happens mainly when these redox carriers are charged enough with electrons and the potential energy for transfer is elevated, like in the case of high mitochondrial membrane potential. In contrast, ROS generation is decreased if there are not enough electrons and the potential energy for the transfer is not sufficient (reviewed in Lin and Beal, 2006).

Cells are also able to detoxify the generated ROS due to an extensive antioxidant defence system that includes superoxide dismutases, glutathione peroxidases, catalase, thioredoxins, and

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

A number of proteins bound to the mitochondria or endoplasmic reticulum (ER), especially in the mitochondria-associated ER membrane (MAM) are playing an important role of communicators between these two organelles (reviewed Mei et al., 2013). ER stress induces mitochondrial dysfunction through regulation of Ca^{2+} signaling and ROS production (reviewed Mei et al., 2013). Prolonged ER stress leads to release of Ca^{2+} at the MAM and increased Ca^{2+} uptake into the mitochondrial matrix, which induces Ca^{2+}-dependent mitochondrial outer membrane permeabilisation and apoptosis. At the same, ROS are produced by proteins in the ER oxidoreductin 1 (ERO1) family. ER stress activates ERO1 and leads to excessive production of ROS, which, in turn, inactivates SERCA and activates inositol-1,4,5- trisphosphate receptors (IP3R) via oxidation, resulting in elevated levels of cytosolic Ca^{2+}, increased mitochondrial uptake of Ca^{2+}, and ultimately mitochondrial dysfunction. Just as ER stress can lead to mitochondrial dysfunction, mitochondrial dysfunction also induces ER Stress (reviewed Mei et al., 2013). For example, nitric oxide disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca^{2+} flux which induce ER stress. Increased Ca^{2+} flux triggers loss of mitochondrial membrane potential (MMP), opening of mitochondrial permeability transition pore (MPTP), release of cytochrome c and apoptosis inducing factor (AIF), decreasing ATP synthesis and rendering the cells more vulnerable to both apoptosis and necrosis (Wang and Qin, 2010).

Summing up: Mitochondria play a pivotal role in cell survival and cell death because they are regulators of both energy metabolism and apoptotic/necrotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002; Lee and Wei, 2012; Wang et al., 2011). The production of ATP via oxidative phosphorylation is a vital mitochondrial function (Kann and Kovács, 2007; Nunnari and Suomalainen, 2012). The ATP is continuously required for signalling processes (e.g. Ca^{2+} signalling), maintenance of ionic gradients across membranes, and biosynthetic processes (e.g. protein synthesis, heme synthesis or lipid and phospholipid metabolism) (Kang and Pervaiz, 2012), and (Green, 1998; McBride et al., 2006). Inhibition of mitochondrial respiration contributes to various cellular stress responses, such as deregulation of cellular Ca^{2+} homeostasis (Graier et al., 2007) and ROS production (Nunnari and Suomalainen, 2012; reviewed Mei et al., 2013; Li et al., 2003). It is well established in the existing literature that mitochondrial dysfunction may result in: (a) an increased ROS production and a decreased ATP level, (b) the loss of mitochondrial protein import and protein biosynthesis, (c) the reduced activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, (d) the loss of the mitochondrial membrane potential, (e) the loss of mitochondrial motility, causing a failure to re-localize to the sites with increased energy demands, (f) the destruction of the mitochondrial network, and (g) increased mitochondrial Ca^{2+} uptake, causing Ca^{2+} overload (reviewed in Lin and Beal, 2006; Graier et al., 2007), (h) the rupture of the mitochondrial inner and outer membranes, leading to (i) the release of mitochondrial pro-death factors, including cytochrome c

(Cyt. c), apoptosis-inducing factor, or endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013), which eventually leads to apoptotic, necrotic or autophagic cell death (Wang and Qin, 2010). Due to their structural and functional complexity, mitochondria present multiple targets for various compounds.

How it is Measured or Detected

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

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

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

2. Mitochondrial membrane potential (Δψm)
The mitochondrial membrane potential (Δψm) is the electric potential difference across the inner mitochondrial membrane. It requires a functioning respiratory chain in the absence of mechanisms that dissipate the proton gradient without coupling it to ATP production. The classical and still most quantitative method uses a tetraphenylphosphonium ion (TPP+)-sensitive electrode on suspensions of isolated mitochondria. The Δψm can also be measured in live cells by fluorimetric methods. These are based on dyes which accumulate in mitochochondria because of Δψm. Frequently used are tetramethylrhodamineethylester (TMRE), tetramethylrhodamine, methyl ester (TMRM) (Petronilli et al., 1999) or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1). Mitochondria with intact membrane potential concentrate JC-1, so that it forms red fluorescent aggregates, whereas de-energized mitochondria cannot concentrate JC-1 and the dilute dye fluoresces green (Barrientos and Moraes, 1999). Assays using TMRE or TMRM measure only at one wavelength (red fluorescence), and depending on the assay setup, de-

energized mitochondria become either less fluorescent (loss of the dye) or more fluorescent (attenuated dye quenching).

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

4. ATP content
For the evaluation of ATP levels, various commercially-available ATP assay kits are offered (e.g. Sigma, http://www.abcam.com/atp-assay-kit-colorimetricfluorometric-ab83355.html), based on luciferin and luciferase activity. For isolated mitochondria various methods are available to continuously measure ATP with electrodes (Laudet et al., 2005), with luminometric methods, or for obtaining more information on different nucleotide phosphate pools (e.g. Ciapaite et al., 2005).

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

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

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

3. Generation of ROS and resultant oxidative stress
a. general approach Electrons from the mitochondrial ETS may be transferred ‘erroneously’ to molecular oxygen to form superoxide anions. This type of side reaction can be strongly enhanced upon mitochondrial damage. As superoxide may form hydrogen peroxide, hydroxyl radicals or other reactive oxygen species, a large number of direct ROS assays and assays assessing the effects of ROS (indirect ROS assays) are available (Adam-Vizi, 2005; Fan and Li 2014). Direct assays are based on the chemical modification of fluorescent or luminescent reporters by ROS species. Indirect assays assess cellular metabolites, the concentration of which is changed in the

presence of ROS (e.g. glutathione, malonaldehyde, isoprostanes,etc.) At the animal level the effects of oxidative stress are measured from biomarkers in the blood or urine.

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

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

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

e. Detection of hydrogen peroxide (H_2O_2) production. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products in the presence of hydrogen peroxide (Zhou et al., 1997; Ruch et al., 1983). The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H2O2 form increasing amounts of fluorescent product (Tarpley et al., 2004).

Summing up mitochondrial dysfunction can be measured by: • ROS production: superoxide (O2-), and hydroxyl radicals (OH−) • Nitrosative radical formation such as ONOO− or directly by: • Loss of mitochondrial membrane potential (MMP) • Opening of mitochondrial permeability transition pores (MPTP) • ATP synthesis • Increase in mitochondrial Ca^{2+} • Cytochrome c release

• AIF (apoptosis inducing factor) release from mitochondria • Mitochondrial Complexes enzyme activity • Measurements of mitochondrial oxygen consumption • Ultrastructure of mitochondria using electron microscope and mitochondrial fragmentation measured by labelling with DsRed-Mito expression (Knott et al., 2008) Mitochondrial dysfunction-induced oxidative stress can be measured by: • Reactive carbonyls formations (proteins oxidation) • Increased 8-oxo-dG immunoreactivity (DNA oxidation) • Lipid peroxidation (formation of malondialdehyde (MDA) and 4- hydroxynonenal (HNE) • 3-nitrotyrosine (3-NT) formation, marker of protein nitration • Translocation of Bid and Bax to mitochondria • Measurement of intracellular free calcium concentration ($[Ca^{2+}]i$): Cells are loaded with 4 μM fura-2/AM). • Ratio between reduced and oxidized form of glutathione (GSH depletion) (Promega assay, TB369; Radkowsky et al., 1986) • Neuronal nitric oxide synthase (nNOS) activation that is Ca^{2+}-dependent All above measurements can be performed as the assays for each readout are well established in the existing literature (e.g. Bal-Price and Brown, 2000; Bal-Price et al., 2002; Fujikawa, 2015; Walker et al., 1995).

Evidence Supporting Taxonomic Applicability

Name	Scientific Name	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI

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

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4. Cell injury/death, N/A

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KE	Strong
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KE	Strong
Protein Alkylation leading to Liver Fibrosis	KE	Strong
Binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development can trigger neuroinflammation and lead to neurodegeneration	KE	Moderate
Lysosomal damage leading to liver inflammation	KE	Strong

How this Key Event works

Level of biological organisation
Cellular

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 characterised 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 cytocrome c in the case of Bax. tAIF translocates to cell nuclei, and together with cyclophilin A and phosphorylated histone H2AX (γH2AX) is responsible for DNA cleavage, a feature of programmed necrosis. Activated calpain I has also been shown to cleave the plasma membrane Na^{+}–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 (Fujikawa, 2015). Intracellular stress either directly affects mitochondria or can lead to effects on other organelles, which then send signals to the mitochondria to recruit participation in the death process (Fujikawa, 2015; Malhi et al., 2010). Constitutively expressed nitric oxide synthase (nNOS) is a 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 $ONOO^{-}$, $O2^{-}$ and hydroxyl radical (OH^{-}) damage cellular membranes and intracellular proteins, enzymes and DNA (Fujikawa, 2015; Malhi et al., 2010; Kaplowitz, 2016; Kroemer et al., 2009).

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 synthesised NADH in the presence of an electron acceptor. The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to enumerate the percentage of necrotic cells in a sample (Chan et al., 2013).

The MTT assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Other closely related tetrazolium dyes 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 (Berridge et al., 2005).

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

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

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

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

Evidence Supporting Taxonomic Applicability

Name	Scientific Name	Evidence	Links
human	Homo sapiens	Strong	NCBI
human and other cells in culture		Strong	**Error! Hyperlink reference not valid.**
Rattus norvegicus	Rattus norvegicus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI

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

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5. Neurodegeneration, N/A

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development can trigger neuroinflammation and lead to neurodegeneration	AO	
Binding to SH/selen-proteins can trigger neuroinflammation leading to neurodegeneration	AO	
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KE	Strong

How this Key Event works

Level of biological organisation
Tissue

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, 2008; Robertson et al, 1987), or mercury exposure during the developmental period (Falluel-Morel et al, 2012). Pb^{2+} 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 Pb^{2+} 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 Pb^{2+} (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 recognised, but a causal relationship has not been unequivocally established (Lansbury and Lashuel, 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 diseases (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 (Kaidery et al., 2013).

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 Tuszynski, 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).

Evidence Supporting Taxonomic Applicability

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).

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6. Neuroinflammation, N/A

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding to SH/selen-proteins can trigger neuroinflammation leading to neurodegeneration	KE	Strong
Binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development can trigger neuroinflammation and lead to neurodegeneration	KE	Moderate
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KE	Weak
Inhibition of Complex I of the mitochondrial respiration chain leading to neurodegeneration.	KE	Moderate

How this Key Event works

Level of biological organisation
Tissue

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 and Streit, 1990; Monnet-Tschudi et al., 2007; Streit et al., 1999; Kraft and Harry, 2011; Claycomb et al., 2013). Upon stimulation by cytokines or inflammogens (e.g. from pathogens or from damaged neurons), both glial cell types activate inflammatory signalling pathways, which result in increased expression and/or release of inflammatory mediators such as cytokines, eicosanoids, and metalloproteinases (Dong and Benveniste, 2001), as well as in the production of reactive oxygen (ROS) and nitrogen species (RNS) (Brown and Bal-Price, 2003). Different types of activation states are possible for microglia and astrocytes, resulting in different responses concerning pro-inflammatory/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 lead to astrocyte activation (Falsig, 2008). Two distinct states of microglial activation have been described (Gordon, 2003; Kigerl et al., 2009; Maresz et al., 2008; Mosser and Edwards, 2008; Perego 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 characterised by increased expression of integrin alpha M (Itgam) and CD86, as well as the release of pro-

inflammatory cytokines (TNF-alpha, IL-1beta, IL-6), and it is mostly associated with neurodegeneration. The M2 state is triggered by IL-4 and IL-13 (Maresz et al., 2008; Perego et al., 2011; Ponomarev et al., 2007) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes. The activation of astrocytes by microglia-derived cytokines or TLR agonists resembles the microglial M1 state (Falsig, 2006).

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 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 neuroinflamatory 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, of by measurement of the respective mediators, e.g. by ELISA-related immuno-quantification. Such markers include: • Pro- and anti-inflammatory cytokine expression (IL-1β; TNF-α, Il-6, IL-4) ; or expression of immunostimmulatory 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 examples using the KE:
Measurement of glial fibrillary acidic protein (GFAP), whose increase is a marker of astrocyte reactivity, is required by the US EPA for fuel additives (40 CFR 79.67), but is optional for other toxicant evaluation.

Evidence Supporting Taxonomic Applicability

Name	Scientific Name	Evidence	Links
rat	Rattus sp.	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
human	Homo sapiens	Moderate	NCBI

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: Vennetti 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

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7. Neuronal network function in adult brain, Decreased

Key Event Overview

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KE	Moderate

How this Key Event works

Biological state:

In the brain, neurons never work alone. They create a network where the activity of one cell directly influences many others. Each neuron is a specialised cell and when activated, it fires an electrochemical signal along the axon. A neuron fires only if the total signal received at the cell body from the dendrites exceeds a certain level (the firing threshold). The strength of the signal received by a neuron (and therefore its chances of firing) critically depends on the efficacy of the synapses. Each synapse actually contains a synaptic cleft with neurotransmitter that transmits a signal across the gap. During synaptic transmission neurotransmitters are released by a presynaptic neuron and bind to and activate the receptors of the postsynaptic neuron in response to a threshold of action potential. Synaptic transmission relies on: the availability of the neurotransmitter; the release of the neurotransmitter by exocytosis; the binding of the postsynaptic receptor by the neurotransmitter; the functional response of the postsynaptic cell; and the subsequent removal or deactivation of the neurotransmitter. Neurons form complex networks of synapses through which action potentials travel. When the nerve impulse arrives at the synapse, it may cause the release of neurotransmitters, which influence another (postsynaptic) neuron. The postsynaptic neurons receive inputs from many additional neurons, both excitatory and inhibitory. The excitatory and inhibitory influences are summed (neural summation) resulting in inhibition or "firing" (i.e., generate an action potential) if the threshold potential has been reached. The voltage at which an action potential is triggered happens if enough voltage-dependent sodium channels are activated and the net inward sodium current exceeds all outward currents (Kolb and Whishaw, 2003). Therefore, at the beginning of the action potential, the Na^+ channels open and Na^+ moves into the axon, causing depolarisation. Re-polarisation occurs when the K^+ channels open and K^+ moves out of the axon. This creates a change in polarity between the outside of the cell and the inside. The impulse travels down from the axon hillock in one direction only, to the axon terminal. Here, the neurotransmitter is released releasing neurotransmitter at the synaptic cleft to pass along information to another adjacent neuron. Excitatory inputs bring a neuron closer to a firing threshold, while inhibitory inputs bring the neuron farther from threshold. An action

potential is an "all-or-none" event; neurons whose membranes have not reached threshold will not fire, while those that do, will fire. One of the most influential researchers into neurological systems (Donald Hebb) postulated that learning consisted principally in altering the "strength" of synaptic networking. Recent research in cognitive science, in particular in the area of non-conscious information processing, have further demonstrated the enormous capacity of the human mind to learn simple input-output co-variations from extremely complex stimuli. Consequently, the neurodegeneration and cell death disrupt the natural rhythms of brain network communication. Cognitive disorders are primarily associated with dysfunction of the neurons of the prefrontal cortex, hippocampus and with changes mainly in NMDARs function (Wang and Arnsten, 2015).

Biological compartments:

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

General role in biology:

Glutamatergic neurotransmission (NMDA, AMPA and KA receptors)

The network of glutamatergic neurons is heavily involved in long-term synaptic plasticity, the main process linked to learning and memory. At the same time over-activation of these neurons (excitotoxicity) leads to neuronal cell death that can be mediated by increased levels of extracellular glutamate or a molecule that behaves as its analogue. Glutamate acts at a variety of ionotropic receptors, including AMPARs, kainate receptors, and NMDARs. The NMDARs have been of particular interest due to their unique properties. They require neuronal depolarisation to relieve their Mg^{++} block, and are permeable to Ca^{++} that can initiate second-messenger signalling events, such as mediating neuroplasticity or negative feedback through Ca^{++}-sensitive K^{+} channels. There have been extensive studies on the glutamate NMDAR and AMPAR mechanisms underlying long-term synaptic plasticity in the primary visual cortex and in CA1 neurons of the hippocampus (Liu et al., 2004; Cho et al., 2009; Lüscher and Malenka, 2012). Neuronal network function and long-term plasticity is also regulated by the levels of AMPAR expression as the number of AMPARs inserted into the post-synaptic density can mediate the degree of spine

depolarisation and thus the NMDAR opening. Synaptic plasticity in the mature visual cortex appears to be governed by GluN2A subunits, which have faster kinetics than GluN2B. GluN2B receptors are expressed in synapses early in development, but many move to extra-synaptic locations in the mature visual cortex and hippocampus (Goebel-Goody et al., 2009). The actions of NMDARs on the dorsolateral prefrontal cortex neuronal circuitry network underlying spatial working memory in primates and it mechanism is described in detail by Wang and Arnsten (2015). In the hippocampus, there is some evidence that long-term potentiation (LTP) is mediated by synaptic GluN2A, while long-term depression is mediated by extrasynaptic GluN2B receptors (Liu et al., 2004). Kainate receptors (KARs) also play an important role in neuronal network function. They play a major function in the pre-synaptic terminal, in particular in the hippocampus. Activation of kainate receptors in have been shown to regulate glutamate release (Jane et al., 2009) and to both depress and factilitate transmission in different synapses. Pre-synaptic kainate receptors in the hippocampus facilitate AMPA and NMDA receptor-mediated transmission at mossy fibre-CA3 synapses (Lauri et al., 2005). Activation of post-synaptic KARs facilitates activation of NMDARs as it has been described in the context of DomA exposure.

Role of other neurotransmitters

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

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

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

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

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

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

How it is Measured or Detected

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

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

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

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

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

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

6. Seed-based correlative analysis of [18F]fluorodeoxyglucose (FDG)-PET (FDG-PET) differences in images (resting state minus activation) is suitable to identify cerebral networks in rats. Using awake and freely moving animals enables functional network analysis of complex behavioral paradigms (Rohleder et al., 2015).
Although most experiments at present are carried out in anesthetized animals, several approaches for imaging in awake behaving animals have been devised that ultimately aim at directly correlating neuronal network dynamics with behaviour (Dombeck et al., 2007, Arenkiel et al., 2007). Finally, through expression of light-activated channel proteins, it might become possible in the future to not only read-out but also control neuronal networks in vivo (Garaschuk et al., 2006)since with the development of X-ray, CT, and MRI, deep neural networks involved in learning and memory processes can be studied in vivo (Cheng et al., 2014).

7. NMDAR overactivation-induced LTD that decrease number of spine density can be measured in vitro using GFP technology and by cofilin-F-actin quantification (Calabrese et al., 2014).
Current behavioural tests used for evaluating neural network function:

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

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

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

4. Spontaneous alternation: spontaneous alternation is spatial alternation and represents a tendency to avoid stimulus re-exposure during exploratory behaviour. T-maze (simple or multiple), Y-maze, and radial maze are used to quantify an innate, unlearned response in rats.
These four behavioural tests are described in detail in the review by Myhrer (Myhrer et al., 2003).

Evidence Supporting Taxonomic Applicability

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

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Adverse Outcome

Adverse Outcome
Learning and memory, Impairment

Learning and memory, Impairment

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	AO	
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	AO	
Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	AO	
Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KE	
Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure	KE	

How this Key Event works

Level of biological organisation
Individual

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 behavioural 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; Gilbert et al., 2012). 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 (Burrone 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 characterised 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 neudegenerative 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 and Iliffe, 2009).

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 characterised 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.

Evidence Supporting Taxonomic Applicability

Name	Scientific Name	Evidence	Links
human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
fruit fly	Drosophila melanogaster	Strong	NCBI
zebrafish	Danio rerio	Strong	NCBI
gastropods	Physa heterostropha	Strong	NCBI

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).

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Key Event Relationships: Scientific evidence supporting the linkages in the AOP

Event	Description	Triggers
Inotropic glutamate receptors, Binding of agonist	Directly Leads to	NMDARs, Overactivation
NMDARs, Overactivation	Directly Leads to	Calcium influx, Increased
Calcium influx, Increased	Indirectly Leads to	Mitochondrial dysfunction, N/A
Mitochondrial dysfunction, N/A	Indirectly Leads to	Cell death, N/A
Cell death, N/A	Directly Leads to	Neurodegeneration, N/A
Cell death, N/A	Indirectly Leads to	Neuroinflammation, N/A
Neurodegeneration, N/A	Directly Leads to	Neuroinflammation, N/A
Neuroinflammation, N/A	Directly Leads to	Neurodegeneration, N/A
Neurodegeneration, N/A	Indirectly Leads to	Neuronal network function in adult brain, Decreased
Neuronal network function in adult brain, Decreased	Indirectly Leads to	Learning and memory, Impairment

1. Inotropic glutamate receptors, Binding of agonist leads to NMDARs, Overactivation

How Does This Key Event Relationship Work

NMDARs can be activated indirectly through initial activation of KA/AMPARs as it happens in the case of DomA exposure. DomA is an agonist of presynaptic and postsynaptic KARs and sustained activation of these receptors by DomA results in massive ion flux and excessive release of glutamate from excitatory terminals causing depolarisation of the postsynaptic neuron (as descibed in MIE). Upon this depolarisation the Mg^{2+} block is removed from the pore of NMDARs, resulting in their activation allowing sodium, potassium, and, importantly, calcium ions to enter into a cell. The sustained exposure to DomA causes pathological overactivation of

NMDARs. In the case of exposure to glufosinate NMDARs activation is triggered by direct, sustained binding of glufosinate to the NMDARs.

Weight of Evidence

Biological Plausibility

NMDARs are unique among ligand-gated ion channels in that their activation requires binding of two co-agonists, glycine and endogenous neurotransmitter, L-glutamate. Physiologically, however, glycine and glutamate have distinct functions. While L-glutamate is released from specific presynaptic terminals, low concentrations of ambient glycine present at the synapse are thought to be sufficient to allow receptor activation. There is a clear understanding that binding of glutamate or its analogue will activate NMDA receptor (accepted dogma). The prolonged activation of NMDARs will lead to a pathological over-activation of a receptor leading to excitotoxicity (minor role of KA/AMPARs), allowing high levels of calcium ions to enter the cell. However, KA/AMPARs play an important role for indirect NMDAR activation since (almost always) an initial activation of these receptors triggers depolarisation of postsynaptic neurons that relieves the block of the channel pore by Mg^{2+}, resulting in NMDAR activation. NMDA receptors are formed by a ligand binding domain (LBD) and an ion channel that are considered the core structural and functional elements of the receptors. There is a clear understanding of how agonist binding leads to channel opening that relies on structural (e.g. crystallography or NMR) and functional (e.g. UV and IR spectrometric measurements) experimental studies of the water-soluble LBD combined with functional studies of the intact receptor. After the initial agonist binding, a conformational change - so-called clam shell closure - that prevents agonist dissociation occurs followed by a conformational change in the ion channel that is tightly coupled to that in the LBD (reviewed in Traynelis et al., 2010). Consequently it can be stated that there is a clear structural and functional mechanistic understanding in this KER between MIE (Binding of agonist to glutamate ionotropic receptors) and KE1, NMDAR overactivation that, as explained above, can be triggered by direct binding to NMDAR or indirectly, through initial activation of KA/AMPARs as it happens in the case of exposure to glufosinate and DomA respectively, two stressors described in this AOP.

Indeed, domoic acid has a very strong affinity for the ionotropic glutamate receptors, the activation of which results in excitotoxicity, initiated by an integrative action of ionotropic receptors at both sides of the synapse blocking the channel from rapid desensitisation. It has a synergistic effect with endogenous glutamate and it acts mainly as an agonist for presynaptic and postsynaptic kainate receptors. Activation of ionotropic receptors leads to the influx of Na^{+}, K^{+} and Ca^{2+}, particulary after activation of NMDARs. In combination with the inhibitory GABA neurotransmitter, glutamate contributes to the control of overall neuronal excitability.

Gufosinate (GLF) triggers alterations in glutamatergic signaling through direct binding and activation of NMDARs (Lantz et al., 2014: Matsumura et al., 2001). GLF agonist action at the NMDAR is expected to occur through interaction with the glutamate binding site and requires binding of the glycine co-agonist as well as release of the magnesium block from the channel pore. Additionally, the possible inhibition by GLF of the high affinity glutamate re-uptake transporter, especially GLT-I was studied to determine whether GLF could increase the levels of endogenous glutamate at the synaptic cleft, resulting in over activation of NMDARs. Such

mechanism was excluded by Lantz (Lantz et al., 2014) but suggested by other studies (Watanabe and Sano, 1998).

Empirical support for linkage

There is well established understanding of NMDAR activation by endogenous agonist glutamate that happens in the absence of the Mg^{2+} block under conditions of depolarized post-synaptic membrane (accepted dogma) (Blanke et al., 2009a and b; Enoki R, et al., 2004).

Single channel behavior of NMDARs from hippocampal CA1 neurons was studied using very low glutamate concentrations to improve temporal resolution of individual glutamate binding events. Openings resulting from individual receptor activations showed surprising complexity: they consist of a long cluster of bursts of openings. Furthermore, the NMDARs appeared to have different gating modes, occasionally entering periods of very high open probability (Gibb and Colquhoun, 1991). Single channel analysis also provided insight in how NMDARs function at the synapse. In response to a brief pulse of glutamate, mimicking synaptic release, NMDARs activate slowly over hundreds of milliseconds and continue activating long after all glutamate has been removed from the synaptic cleft, thereby briefly "memorising" the occurrence of a synaptic input. Single channel analysis of NR1 and NR2A receptors indicates that after a brief pulse of glutamate, receptors enter a high affinity closed state from which either channel opening or agonist unbinding occurs with approximately equal probability (Popescu et al., 2004). A single synaptic event is therefore expected to only partially activate NMDARs. Consequently, a closely spaced second pulse of agonist is able to further increase the open probability, endowing the NMDAR with an ability to decode synaptic input frequency.

Domoic acid is an agonist for presynaptic and postsynaptic kainate receptors, however indirectly also activates NMDA receptors. Kainate receptors are localised both at presynaptic and postsynaptic sites. At presynaptic sites, they directly affect transmitter release from both excitatory and inhibitory neuron terminals. At postsynaptic sites, kainate receptors lead to cell depolarisation, which would bring the neuron closer to its spike firing threshold. By having this dual localisation, kainate receptors help in the control of neuronal excitability. However, sustained activation of postsynaptic kainate receptors by domoic acid results in massive ion flux and excessive release of glutamate from excitatory terminals. The released glutamate in turn activates NMDA receptors, which have lost their physiologic Mg^{2+} block because of domoic acid–induced depolarisation. The final event is an increase of NMDA-mediated Ca^{2+} flux and subsequent activation of intracellular pro-oxidative cascades and ion imbalances, eventually leading to excitotoxicity-mediated neuronal death (Babot et al., 2005; Giordano et al., 2006).

Kainate receptors are widely expressed in the hippocampus. Glutamatergic granule cells in the hippocampus express these receptors, suggesting that cell death found after domoic acid intoxication may be produced by hyperstimulation of NMDA receptor after glutamate is released in excess. In agreement with this hypothesis, the seriously damaged CA3 area of the hippocampus receives projections from hippocampal granule cells. Qiu and Curras-Collazo (Qiu et al., 2006a) elegantly demonstrated that domoic acid first targets kainate receptors in the hippocampus by blocking its effects in vivo with a kainate receptor antagonist. The sequential involvement of distinct glutamate receptors was confirmed and further elucidated in rat mixed cortical cell and hippocampal slice cultures (Jakobsen et al., 2002; Qiu et al., 2006b).

Using primary cultures of rodent cerebellar granule cells, an in vitro model mainly constituted by glutamatergic neurons that express both NMDA and kainate receptors it was proved that domoic acid increased glutamate release, intracellular calcium, and cell death, which were prevented by kainate and NMDA receptor antagonists (Berman and Murray, 1997; Vale-Gonzalez et al., 2006) confirming that DomA toxicity is mediated by both KA and NMDARs.

Glufosinate (GLF) and its primary metabolite N-acetylglufosinate (NAcGLF) interaction with NMDARs was studied in the primary culture of rat cortical neurons by performing [3H]CGP 39653 binding experiments. The results showed that their binding affinity to NMDAR (IC50, GLF 668 uM and NAcGLF approximately 100 uM) corresponded to the concentration that produce the highest increase of mean firing rate. Furthermore, they produced biphasic MFR profile, specific to NMDAR agonists. The obtained results suggest that GLF and NAcGLF can produce both effects, excitatory and inhibitory on network activity through direct activation of NMDARs (Lantz et al., 2014).

Direct activation of NMDARs by GLF is also suggested by in vivo studies where three NMDA receptor antagonists, dizocilpine, LY235959, and Compound 40, and AMPA/KA antagonist, NBQX, were co-administrated with glufosinate ammonium (80 mg/kg, intraperitoneally) in mice. Statistical analyses showed that the NMDA receptor antagonists markedly inhibited the GLF-induced convulsions, while the AMPA/KA receptor antagonist had no effect. These results suggest that the convulsion caused by glufosinate ammonium were mediated through activation of NMDA receptors (Matsumura et al., 2001).

Uncertainties or Inconsistencies

The increase in MFR induced by GLF in neuronal networks was significantly blocked by MK-801 but not entirely suggesting that GLF can increase activity in the MEA system through non-synaptic NMDARs, since these are not blocked by MK-801. It is not entirely clear whether GLF can work through an inhibition of the glutamate reuptake transporter, GLT-I, increasing the concentration of endogenous glutamate at the synaptic cleft and subsequently resulting in over activation of NMDARs (Lantz et al., 2014: Watanabe and Sano, 1998). Further studies are necessary to determine whether this alternative mechanism of GLF-induced NMDAR overactivation takes place. Additionally GLF also modulates glutamine synthetase (GS) activity. Since, astrocytic GS in the brain participates in the metabolic regulation of glutamate (endogenous agonist of NMDAR) it is not clear if this pathway contributes to NMDAR activation too.

Quantitative Understanding of the Linkage

To predict how potent an agonist can be, it is usually based on the half maximal effective concentration (EC50) that induces the currents through NMDA receptors of brain slices and cells (or in recombinantly expressed proteins of these receptors). Traynelis et al. (2010) summarised the IC50 values for agonists of the different NMDA receptor subunits. The activation effect (efficacy) of agonist on NMDA receptor have been found to be dependent on: -the type of subunits that form the NMDA receptor -the chemical structure of the agonist -the binding site of a receptor that the agonist prefers -how tightly an agonist binds to the receptor (affinity) Glufosinate

and its primary metabolite N-acetylglufosinate NAcGLF bind to the NMDAR with the following affinity: the IC50 value for GLF was 668 mM and for NAcGLF was about 100 mM.

Evidence Supporting Taxonomic Applicability

Various studies suggest the existence of functional NMDA-like receptors in invertebrates (Xia et al., 2005). Fly and rodent NMDARs exhibit several important differences (Murphy and Glanzman, 1997). The expression and function of NMDA receptors in rodent and primates is well characterised in the existing literature.

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2. NMDARs, Overactivation leads to Calcium influx, Increased

How Does This Key Event Relationship Work

The NMDA receptor is distinct from the other glutamate receptors in two ways: first, it is both ligand-gated and voltage-dependent; second, it requires co-activation by two ligands: glutamate (GLU) and either glycine or D-serine. Following membrane depolarisation, the co-agonists, L-glutamate and glycine must bind to their respective sites on the receptor to open the channel. On activation, the NMDA receptor allows the influx of extracellular calcium ions into the postsynaptic neuron and neurotransmission occurs (reviewed in Higley and Sabatini, 2012). Calcium flux through NMDA receptors is also thought to be critical in synaptic plasticity, a cellular mechanism for learning and memory. Indeed, NMDA receptor–dependent synaptic potentiation (LTP) and depression (LTD) are two forms of activity-dependent long-term changes in synaptic efficacy that are believed to represent cellular correlates of learning and memory processes. The best characterised form of NMDA receptor-dependent LTP and LTD occurs between CA3 and CA1 pyramidal neurons of the hippocampus (Luscher and Malenka, 2012). It is now well established that modest activation of NMDARs leads to modest increases in postsynaptic calcium, triggering LTD, whereas much stronger activation of NMDARs leading to much larger increases in postsynaptic calcium are required to trigger LTP (Luscher and Malenka, 2012). The high-frequency stimulation causes a strong temporal summation of the excitatory postsynaptic potentials, and depolarisation of the postsynaptic cell is sufficient to relieve the Mg^{2+} block of the NMDAR and allow a large amount of calcium to enter into the post-synaptic cells.

Weight of Evidence

Biological Plausibility

There is structural and functional mechanistic understanding supporting this relationship between KE NMDARs, Overactivation and KE Calcium influx, Increased.

This relationship is plausible as the expression of the functional NMDA receptors is commonly carried out or assessed by Ca^{2+} imaging method. Calcium imaging techniques have been extensively utilised in the literature to investigate the potential interactions between NMDA-evoked Ca^{2+} influx and NMDA receptor activation. Approximately 15% of the current through NMDA receptors is mediated by Ca^{2+} 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 Ca^{2+} influx of about 6000 ions into a spine head reaching a concentration of ~10 µm (Higley and Sabatini, 2012). However, the majority of the ions are rapidly eliminated by binding to Ca^{2+} proteins, reaching ~1 µM of free Ca^{2+} concentration (Higley and Sabatini, 2012).

It has been shown that in rat primary forebrain cultures the intracellular Ca^{2+} increases after activation of the NMDA receptor through administration of NMDA but this increase in Ca^{2+} is blocked when the cells are cultured under Ca^{2+} free conditions, demonstrating that the NMDA-evoked increase in intracellular Ca^{2+} derives from extracellular and not intracellular sources (Liu et al., 2013).

Indirect mechanism of domoic acid (DA) induced overactivation of NMDARs that result in Ca^{2+} overload: depolarisation of the pre-synaptic cell activates the release of endogenous Ca^{2+} which mobilises vesicles containing GLU to the membrane surface. Glutamate is then released into the synaptic cleft by exocytosis where it is able to interact with cell surface receptors. Exogenous DA can interact within the synaptic cleft with each of the three ionotropic receptor subtypes including the kainate, AMPA, and NMDA receptors on cell membranes. Activation of the kainate and AMPA receptors results in release of Ca^{2+} via coupled ion channels, into the post-synaptic cell. DA is also able to bind to NMDA receptors that are linked to both Ca^{2+} and Na/K^{+} ion channels and results in a cellular influx of both Na^{+} and Ca^{2+}. Unlike GLU, DA induces prolonged receptor activation causing a constant influx of cations into the cell and the appropriate chemical cues for desensitization are blocked. The excess intracellular Ca^{2+} causes disruption of cellular function, cell swelling and ultimately cell death (Lefebvre and Robertson, 2010).

Glufosinate (GLF) is the methylphosphinate analog of glutamate that directly can activate NMDARs (Lantz et al., 2014, Matsumura et al., 2001, Faro et al., 2013) (as described in KE: *NMDARs, Binding of agonist*). It is well established in the existing literature that activation of NMDARs leads to the intra-cellular Ca^{2+} overload and based on this assumption it can be suggested that an exposure to GLF leads to increased intra-cellular calcium levels.

Empirical support for linkage

Domoic acid (DomA)

- Treatment of mouse cerebellar granule neurons (CGNs) with 1 or 10 µM DomA causes increase of intracellular Ca^{2+} by approximately 5 or 8 fold compared to controls, respectively (Giordano et al., 2006). Interestingly, when the cells are exposed simultaneously to DomA and the NMDA receptor antagonist MK-801, the Ca^{2+} levels measured are close to control levels, indicating that the Ca^{2+} elevation evoked by DomA involves activation of NMDA receptors (Giordano et al., 2006).

- The same research group has performed a time course study by applying a high and a low DomA concentration and using CGNs from Gclm (+/+) and Gclm (−/−) mice lacking glutathione (Giordano et al., 2007). The low DomA dose (0.1µM) causes a small and delayed increase in intracellular Ca^{2+} concentration with a full recovery by 20 min. When

the experiment is performed in the absence of extracellular calcium, this increase of intracellular Ca^{2+} levels in the presence of DomA is abolished, indicating that this change in homeostasis of Ca^{2+} is due to ion entry from outside the cell. However, this recording of intracellular Ca^{2+} is antagonised only by NBQX (AMPA receptor antagonist), but not by MK-801 (NMDA receptor antagonist). On the other hand, the higher DomA concentration (10μM) causes a rapid and robust increase in intracellular Ca^{2+}, which lasts even after 25 min. This effect is antagonized by both NBQX and MK-801, suggesting that not only AMPA but also NMDA receptors are involved in Ca^{2+} elevation evoked by DomA at high doses (Giordano et al., 2007).

- In an earlier study, the time course and concentration dependence of the increase in intracellular Ca^{2+} stimulated by DomA has been examined in 10-13 day-in-culture CGNs (Berman et al., 2002). DomA produces a rapid and concentration-dependent increase in intracellular Ca^{2+}, showing the maximal increase at 10 μM DomA (Berman et al., 2002). At this concentration, fluo-3 fluorescence that is used to measure Ca^{2+} elevates rapidly during the first 40 s of exposure, increases more slowly before peaking at 3.5 min, after which the signal diminishes steadily over the 30 min course of the experiment to 55% of peak values. The EC50 for DomA-induced increase in intracellular Ca^{2+} is 0.61 μM. In the same study, the NMDA receptor antagonist MK-801 significantly reduced both peak and final plateau of intracellular Ca^{2+} by 30 and 70%, respectively (Berman et al., 2002).

- These three studies (Giordano et al., 2006; 2007; Berman et al., 2002) do not provide a simultaneous measurement of NMDA receptor activation by DomA and intracellular Ca^{2+} levels. However, they do provide indirect evidence of NMDA receptor activation involvement in increased intracellular Ca^{2+} concentrations induced by DomA as they have used known antagonists of the NMDA receptors that reverses the situation in both KEs (blocking upstream KE will block downstream KE).

- In an in vivo study it was indirectly shown that the microinjection to adult male Sprague Dawley rats of 10 μM DomA increased intracellular Ca^{2+} levels. A significant upregulation of phosphorylated CaMKII and phosphorylated CREB levels was recorded, possibly due to increased intracellular Ca^{2+} levels induced by DomA (Qiu and Currás-Collazo, 2006).

In CGNs, the co-treatment with 10 μM DomA and the kainate/AMPA receptor antagonist NBQX maintains Ca^{2+} levels near to control levels, suggesting that the Ca^{2+} elevation evoked by DomA is mediated by the activation of both AMPA/kainate and of NMDA receptors (Giordano et al., 2006).

The voltage-sensitive Ca^{2+} channel (VSCC) blocker nifedipine (5 μM) and NBQX (10 μM), a competitive AMPA/kainate receptor antagonist reduces the peak and final intracellular Ca^{2+} concentration in CGNs (Berman et al., 2002), strengthening the view that the increase of Ca^{2+} influx is not only mediated by NMDA receptors but also by AMPA/kainate receptors and VSCCs.

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Overactivation of NMDAR (KE up)	Increased intracelllular Ca^{2+} levels (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Mouse cerebellar granule neurons (CGNs) from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		Time course (15 to 120 min)		5 and 8 fold increase of $[Ca^{2+}]i$ compared to controls.	Giordano et al., 2006				The cells were exposed simultaneously to DA and the NMDA receptor antagonist MK-801 and the Ca^{2+} levels were found to be close to control levels, indicating that the Ca^{2+} elevation evoked by DA involves activation of NMDA receptors.
DomA	CGNs from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		Time course (0 to 25 min)		0.1µM domoic acid caused a small and delayed increase (4 fold) in $[Ca^{2+}]i$, with a full recovery by 20 min.In contrast, the higher concentration of domoic acid (10µM) caused a rapid and robust increase (8 fold) in $[Ca^{2+}]i$, which was still elevated after 25 min. 0.1µM DA increases $[Ca^{2+}]M$ by about 3 fold, with a delay of about 15 min. In contrast, no changes in $[Ca^{2+}]M$ were observed following 10µM of DA.	Giordano et al., 2007				At the low concentration (0.1µM), the recording of intracellular Ca^{2+} was antagonized only by NBQX (AMPA receptor antagonist), but not by MK-801 (NMDA receptor antagonist). On the other hand, the higher DA concentration (10µM) caused a rapid and robust increase in intracellular Ca^{2+} . This effect was antagonized by both NBQX and MK-801, suggesting the importance of NMDA receptors in Ca^{2+} elevation evoked by DA but only at high doses

DomA	10-13 DIV CGNs obtained from 8-day-old Sprague–Dawley rats	0.1 to 30 μM		Time course (0 to 45 min)		EC50 for DA-induced increase in intracellular Ca^{2+} was 0.61 μM	Berman et al., 2002				The NMDA receptor antagonist MK-801 significantly reduced both peak and final plateau of intracellular Ca^{2+} by 30 and 70%, respectively
DomA	Adult male Sprague Dawley rats	10 μM	Brain microinjection			Increased phosphorylated CaMKII and phosphorylated CREB levels	Qiu and Currás-Collazo, 2006				

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

Uncertainties or Inconsistencies

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

Quantitative Understanding of the Linkage

The experiments describing semi-quantitative effects for these KERs are described in the table above.

Evidence Supporting Taxonomic Applicability

Data not available

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3. Calcium influx, Increased leads to Mitochondrial dysfunction, N/A

How Does This Key Event Relationship Work

One of the mitochondrial functions is to buffer intracellular Ca^{2+} levels facilitating the maintenance of Ca^{2+} homeostasis in the cell. In the case of Ca^{2+} overload, mitochondria are not able to buffer the excess of Ca^{2+} that leads to mitochondrial dysfunction measured by the increased generation of reactive oxygen species (ROS), triggering mitochondrial permeability transition pore opening (Choi et al., 2013) and reduced ATP production (reviewed in Gleichmann and Mattson, 2011).

Weight of Evidence

Biological Plausibility

There is functional and structural mechanistic understanding supporting the relationship between KE "Ca^{2+} influx, increased" and KE "Mitochondrial dysfunction".

The increase in cytoplasmic Ca^{2+} can cause the activation of plasma membrane and endoplasmic reticulum (ER) Ca^{2+}-ATPases that results in higher ATP demand. At the same time elevated Ca^{2+} can cause reduced levels of ATP by the direct uptake of the cation into the matrix that utilises the proton circuit and directly competes with mitochondrial ATP synthesis (reviewed in Nicholls, 2009).

Ca^{2+} overload besides of being detrimental to mitochondrial energy production can also induce mitochondrial ROS generation. A number of possible mechanisms have been suggested by which Ca^{2+} overload can increase ROS production including: 1) stimulated increase of metabolic rate by Ca^{2+}, 2) stimulated nitric oxide production by Ca^{2+}, 3) Ca^{2+} induced cytochrome c dissociation, 4) Ca^{2+} induced cardiolipin peroxidation, 5) Ca^{2+} induced mitochondrial permeability transition pore (MPTP) opening with release of cytochrome c (leading to apoptosome formation and caspase-3 activation)and apoptosis inducing factor (AIF), decreased level of reduced glutathione (GSH), the antioxidative enzymes, and 6) Ca^{2+}-calmodulin dependent protein kinase activation (reviewed in Peng and Jou, 2010; Gleichmann and Mattson, 2011). It is worth mentioning that mitochondrial ROS increase is capable of modulating Ca^{2+} dynamics causing further increase of Ca^{2+} levels.
The cytoplasmic and mitochondrial Ca^{2+} levels, the oxidative stress and the energy production are very closely inter-related. For example, decreased (or lack) of ATP production can affect the function of plasma membrane Ca^{2+} pump activity causing Ca^{2+} overload, oxidative stress and further restriction in ATP generating capacity (reviewed in Nicholls, 2009). Prolonged oxidative stimuli cause further mitochondrial dysfunction, including the decrease of mitochondrial transmembrane potential ($\Delta\Psi m$), further overload of mitochondrial calcium, and opening of mitochondrial permeability transition pore (MPTP) (Choi et al., 2013).

Mitochondria within dendrites are elongated and perform extensive directional and lateral movement at physiological conditions. Under an excitotoxic exposure to glutamate, mitochondrial movement has been found to be inhibited and mitochondria change morphology becoming rounded and swollen. Although blocking mitochondrial ATP production is sufficient to inhibit mitochondrial movement (Rintoul et al., 2003), research has shown that the collapse of mitochondrial structure requires extracellular Ca^{2+} influx via NMDA receptors (Rintoul et al., 2003; Pivovarova et al., 2004; Shalbuyeva et al., 2006), suggesting that structural, mechanistic understanding is also available supporting this KER.

In neurons, the high mitochondrial content in axons and dendrites closely correlates with the high energy demand in these structures that is needed to pump the ions that underlie the generation of action potentials mediated by the electrochemical gradients (Attwell and Laughlin, 2001).

Empirical support for linkage

Domoic acid (DomA)

- DomA has been shown to cause a significant time- and concentration-dependent increase of ROS production in mouse cerebellar granule neurons (CGNs) and the maximal effect (2.5 fold increase) has been recorded 1 h after exposure (Giordano et al., 2006). The time course involved the measurement of oxidant-sensitive fluorescent dye DCFH2-DA from 15 to 120 min and the concentrations assessed are 1 and 10 μM DomA (Giordano et al., 2006). ROS production is higher in Gclm (-/-) neurons lacking glutathione (21.97 pmol DCF/mg of protein) than in Gclm (+/+) neurons (10.23 pmol DCF/mg of protein) after treatment with DomA (Giordano et al., 2006). In the same study, treatment of mouse CGNs with 1 and 10 μM DomA elevate intracellular Ca^{2+} by approximately 5 or 8 fold higher compared to controls, respectively (Giordano et al., 2006), showing that the cytosolic Ca^{2+} increase (upstream KE) is higher than the down-stream KE (ROS production due to mitochondrial dysfunction).

- The same research group has measured intracellular Ca^{2+} concentrations at different time points after DomA treatment of cerebellar granule neuons (CGNs) from mice lacking the modifier subunit of glutamate-cysteine ligase (Gclm). Glutamate-cysteine ligase (Glc) catalyzes the first and rate-limiting step in glutathione (GSH) biosynthesis. CGNs from Gclm (-/-) mice have very low levels of GSH and are 10-fold more sensitive to DomA-induced toxicity than CGNs from Gclm (+/+) mice (Giordano et al., 2007). The low DomA dose (0.1μM) causes a small and delayed increase in intracellular Ca^{2+} concentration with a full recovery by 20 min, whereas, the higher DomA concentration (10 μM) causes a rapid and robust increase in intracellular Ca^{2+}, which lasts even after 25 min, revealing that upstream KE (cytosolic Ca^{2+}) happens much earlier than the down-stream KE (ROS production). Interestingly, in the same study the mitochondrial Ca^{2+} concentration has been measured and showed that 0.1μM DomA causes an increase by about 3-fold, with a delay of about 15 min, but no changes in mitochondrial Ca^{2+} concentration have been observed at 10 μM of DomA (Giordano et al., 2007).

- Mice injected intraperitoneally (i.p.) at a dose of 2 mg/kg of DomA once a day for 3 weeks show markedly lowered (1.5-2 fold) respiratory control ratio, mitochondrial ATP

production rate, electron transport chain activity and cellular ATP concentration (Lu et al., 2012; Wu et al., 2013). In Lu et al. 2013 the same treatment in mice causes a 3 or 1.8-fold decrease in electron transport chain activity and mitochondrial ATP content, respectively. Western blot analysis demonstrates that the level of complex I-V proteins (mt-Nd6, Sdha, Uqcrc1, mt-Co1, and Atp5a1) in the hippocampus of DomA-treated mice is significantly decreased compared to controls (Lu et al., 2012). In the same study, DomA treatment significantly elevate the expression of NOX subunits (p47phox and gp91phox), of ROS (3.2 fold increase) and protein carbonyl levels, as well as the production of superoxide anion radicals (Lu et al., 2012). Under the same experimental conditions an increase of NOX activity (2 fold) has been reported in the hippocampus of DomA-treated mice (Lu et al., 2013). Furthermore, DomA exposure induces ER stress by increasing the levels of phosphorylated pancreatic endoplasmic reticulum-resident kinase (PERK), eukaryotic translation initiation factor 2α (eIF2α), glucose-regulated protein 78, C/EBP homologous protein (CHOP), X-box binding protein 1 (XBP1) and the phosphorylated inositol-requiring enzyme 1 (IRE1) (Lu et al., 2012).

- DomA (0.75 mg/kg body weight) administered intravenously in adult rats reveals no remarkable changes at the mRNA level of iNOS expression but demonstrates significant induction in the expression of iNOS protein level in the neurons and astrocytes of the hippocampus (Ananth et al., 2003).

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Increased intracelllular Ca^{2+} levels (KE up)	Mitochondrial dysfunction (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Mouse cerebellar granule neurons (CGNs) from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		Time course (15 to 120 min)	5 and 8 fold increase of $[Ca^{2+}]i$ compared to controls.	Increase in ROS production (2.5 fold) after 1 h of exposure.	Giordano et al., 2006		Same dose	Incidence of upstream KE (Increased intracelllular Ca^{2+} levels) is higher than the incidence of downstream KE (mitochondrial dysfunction)	
DomA	CGNs from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		Time course (0 to 25 min)	0.1µM domoic acid caused a small and delayed increase (4 fold) in $[Ca^{2+}]i$, with a full recovery by 20 min. In contrast, the higher concentration of domoic acid (10µM) caused a rapid and robust increase (8 fold) in $[Ca^{2+}]i$, which was still elevated after 25 min. 0.1µM DomA increases $[Ca^{2+}]M$ by about 3 fold, with a delay of about 15 min. In contrast, no changes in $[Ca^{2+}]M$ were observed following 10µM of DomA.	DomA (0.1µM) caused a 3 fold increase in DHR fluorescence, which accumulates in mitochondria and fluoresces when oxidized by ROS or reactive nitrogen species. This occurred between 1 and 2 h and was higher in CGNs from Gclm (−/−) mice.	Giordano et al., 2007	Yes	Same dose		

DomA	Adult mice	2 mg/kg	intraperitoneally (i.p.)	Once a day for 3 weeks		Decreased respiratory control ratio (1.5-2 fold), mitochondrial ATP production rate, electron transport chain activity, cellular ATP concentration, electron transport chain activity (3 fold) and mitochondrial ATP content (1.8 fold).	Lu et al., 2012; Lu et al. 2013; Wu et al., 2013				
DomA	Adult rats	0.75 mg/kg				Induction in the expression of iNOS protein level.	Ananth et al., 2003				

Gap of knowledge: there are no studies available showing that Glufosinate (GLF) increases intra-cellular calcium levels causing mitochondrial dysfunction. Such a mechanism of toxicity can be assumed taking into consideration that GLF neurotoxicity is induced by direct activation of NMDARs.

Quantitative Understanding of the Linkage

It was established that the dendritic calcium levels could underlie the differential vulnerability of C57BL/6 (resistant to kainite excitotoxicity) and C57BL/10 strains (vulnerable) mice to triggered neuronal degeneration induced by increased Ca^{2+} levels (Shuttleworth and Connor, 2001). A striking difference was found in dendrite calcium responses in hippocampus after kainate exposure of C57BL/6 (resistant to kainite excitotoxicity) and C57BL/10 strains (vulnerable). Ca^{2+} signals in distal dendrites were large in C57BL/10 neurons, and, if a threshold concentration of 1.5 uM was reached, a region of sustained high Ca^{2+} was established in the distal dendritic tree. This region then served as an initiation site for a degenerative cascade, producing high Ca^{2+} levels that slowly spread to involve the entire neuron and led to neuronal cell death. Dendritic Ca^{2+} signals in C57BL/6 neurons were much smaller and did not trigger these propagating secondary responses. Neurons from both strains had similar membrane properties and responded to kainate with intense action potential firing. Degenerative Ca^{2+} responses were seen in both strains if soma calcium level was above 1.5 uM serving as a threshold that if exceeded, it triggered excitotoxic neuronal cell death (Shuttleworth and Connor, 2001).

Evidence Supporting Taxonomic Applicability

DomA toxicosis in California sea lions (CSLs, Zalophus californianus) is accompanied by increased expression of markers of oxidative stress such as malondialdehyde (MDA) and 3-nitrotyrosine (NT) in neurons (Madl et al., 2014).
In Atlantic salmon (Salmo salar), the cognition function has been investigated after exposure to sub-lethal doses of DomA (6 mg DA/kg bw). In addition, 14C-2-deoxyglucose has been injected i.m. to measure brain metabolic activity by autoradiography. The three brain regions investigated telencephalon, optic tectum and cerebellum have demonstrated a clear increase of metabolic activity in DomA exposed brains (Bakke and Horsberg, 2007).

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4. Mitochondrial dysfunction, N/A leads to Cell death, N/A

How Does This Key Event Relationship Work

ROS generation is known to activate different pathways leading to apoptosis, whereas depletion of energy production induces necrotic cell death.

Weight of Evidence

Biological Plausibility

There is functional mechanistic understanding supporting this relationship between KE Mitochondrial dysfunction and KE Cell death.
ROS are known to stimulate a number of events and pathways that lead to apoptosis, triggered by ROS-induced ER stress signalling pathway (Lu et al., 2014), caspase-dependent and -independent

apoptosis (Zhou et al., 2015) and mitogen-activated protein kinase (MAPK) signal transduction pathways (reviewed in Cuadrado and Nebreda, 2010; Harper and LoGrasso, 2001).

Depletion of cellular ATP is known to cause switching from apoptotic cell death triggered by a variety of stimuli to necrotic cell death (Leist et al., 1997) suggesting that the level of intracellular ATP determines whether the cell dies by apoptosis or necrosis (Nicotera et al., 1998). There is strong proof that apoptosis requires energy, as it is a highly regulated process involving a number of ATP-dependent steps such as caspase activation, enzymatic hydrolysis of macromolecules, chromatin condensation, bleb formation and apoptotic body formation (Richter et al., 1996).

Empirical support for linkage

Domoic acid (DomA)

In the case of DomA, in vitro studies have shown that oxidative stress and oxidative stress-induced activation of the stress-activated protein kinase/c-jun-N-terminal kinase (SAPK/JNK) pathway is implicated in DomA-mediated apoptosis (Giordano et al., 2007; 2008; 2009; Lu et al., 2010). In vivo findings also show that ROS-mediated cognitive deficits are associated with apoptosis induced by activation of the JNK pathway (Lu et al., 2010; 2011).

- Mice injected intraperitoneally (i.p.) with DomA at a dose of 2 mg/kg once a day for 4 weeks have shown increase (6 fold) of the TUNEL positive cells in the hippocampus. In the same study they have found that indicators of mitochondria function are markedly decreased (1.5-2 fold) and ROS levels are elevated (3.2 fold) (Lu et al., 2012). DomA treatment also significantly decreases the levels of bcl-2, procaspase-3 and procaspase-12 and increases the activation of caspase-3 and caspase-12 in the mouse hippocampus (Lu et al., 2012). The same research group using similar dose but longer exposure (4 weeks), has shown increase of ROS (3 fold) and NOX (2 fold) and elevated (8 fold) mean value of TUNEL-positive cells in the hippocampal CA1 sections as well as increase in the activation of caspase-8 and caspase-3 (Wu et al., 2012). These two in vivo studies (Lu et al., 2012; Wu et al., 2012) suggest that both KEs are affected in response to the same dose of DomA and exposure paradigm and that the incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction).

- The cell viability has been measured by the MTT reduction assay in mouse cerebellar granule neurons (CGNs) and showed that the IC50 values for DomA are 3.4 μM in Gclm (+/+) neurons and 0.39 μM in Gclm (-/-) neurons (Giordano et al., 2006). This reduction in cell viability has been demonstrated to be concentration dependent after studying a range of concentrations of DomA (0.01 and 10 μM). Giordano et al. 2007 have shown that 100 nM DomA induce apoptotic cell death in mouse CGNs. In a follow-up study, the same research group has performed a dose response evaluation and showed that even 50 nM DomA exposure for 1 h (after washout and additional 23 h incubation) can induce apoptosis in CGNs derived from Gclm (+/+) mice, whereas neurons from Gclm (−/−) mice that have very low levels of glutathione are more sensitive as 10 nM DomA induces a significant increase in apoptotic cell number (Giordano et al., 2009). The maximal apoptosis (5 fold compared to controls) in CGNs from both genotypes has been caused by 100 nM DomA. Interestingly, 1 and 10 μM DomA still cause significant apoptosis in both cell types but to a lesser extent compared to 100 nM DomA. ROS have been measured

only at the dose of 100 nM DomA, 30 min after treatment and showed 2.5 fold increase compared to controls in CGNs from Gclm (+/+) mice (Giordano et al., 2009). Caspase 3 activity has also been measured after 12 h with prior 1 h exposure to 100 nM DomA and found to be increased (2.2 fold). In the same study, DomA (100 nM) caused a significant decrease (25%) of Bcl-2 protein levels after 6 h exposure. Again these in vitro studies (Giordano et al., 2007; 2009) suggest that both KEs are affected by the same dose of DomA and that the incidence of KE down (cell death) is higher than the incidence of KE up (mitochondrial dysfunction). Furthermore, KE up (mitochondrial dysfunction) happens earlier (30 min) than KE down (cell death) that takes place 12-24 h later.

- Mixed cortical cultures have been treated with 3, 5, 10, or 50 μM DomA for a variety of exposure durations (10 min, 30 min, 1 h, or 2 h), after which DomA is washed out and the culture medium is replaced with conditioned medium from unexposed sister cultures (Qiu et al., 2006). In all cases neuronal death has been measured 24 h following the beginning of exposure. The results show that DomA-induced neuronal death is determined by both concentration and duration of exposure. After a 10-min exposure, 50 μM DomA produces marked neuronal death of 47.4 %, whereas by 1 h of treatment, the same concentration produces near maximal neuronal death but longer exposures do not increase neuronal death further (Qiu et al., 2006). Regarding time dependence, this study shows that low concentrations of DomA produces more neuronal death if this is measured 22 h after the washout than if measured immediately after DomA treatment, while higher concentrations of DomA (20–100 μM) produces equivalent degrees of neuronal death when measured at these two time points (Qiu et al., 2006). Based on these findings, three EC50 exposure paradigms have been established, which represent weak/prolonged exposure (3 μM/24 h), moderate concentration and duration of exposure (10 μM/2 h), and strong/brief exposure (50 μM/10 min) (Qiu et al., 2006).

- The mean concentration of DomA in rat brain samples obtained at 30 min after intraperitoneal (i.p.) administration of 1 mg/kg DA is 7.2 ng/g (Tsunekawa et al., 2013). These animals have been examined and revealed after histopathological analysis neuronal shrinkage and cell death, including an increase in the percentage of TUNEL positive cells at 24 hours (8.3 %) and after 5 days (19.0 %) compared to the controls (1.7 %) (Tsunekawa et al., 2013). In the same study, indirectly it has been shown that ROS production is associated with these histopathological findings by using the radical scavenger edaravone (Tsunekawa et al., 2013).

- Brain slices from 8-day-old pups have been treated after 2 weeks with 10 μM DomA and assessed with propidium iodine (PI) stain to determine cellular damage (Erin and Billingsley, 2004). A time course has been carried out and viable cultures have been visualized 12, 24, 48 and 92 h after DomA treatment. Changes in PI uptake has been detected after 24 h post-treatment and at 4h the average fold-increase of PI uptake (DomA/control) was 14.5 and 34.5 in cortex and hippocampus, respectively (Erin and Billingsley, 2004). In the same study, incubation of brain slices with DomA induces degradation of α-spectrin to the 120-kDa product after 18 h of treatment but no change has been noted after 12 h incubation, whereas caspase 3 activity results have not been conclusive (Erin and Billingsley, 2004).

- Using observations of neuronal viability and morphology, exposure of cultured murine cortical neurones to DomA for 24 h have shown to induce concentration-dependent neuronal cell death and the EC50 determined to be 75 µM (Larm et al., 1997).

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Mitochondrial dysfunction (KE up)	Cell death (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	16-month-old male ICR mice	2 mg/kg	Intraperitoneally (i.p.)	Once a day for 4 weeks	Indicators of mitochondrial function were markedly decreased (1.5-2 fold) and ROS levels were elevated (3.2 fold).	The mean of TUNEL positive cells in the hippocampus was increased (6 fold). The levels of bcl-2, procaspase-3 and procaspase-12 were significantly decreased and the activation of caspase-3 and caspase-12 in the mouse hippocampus were increased.	Lu et al., 2012		Same dose	Incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction)	
DomA	16-month-old male ICR mice	2 mg/kg	i.p.	Once a day for 4 weeks	ROS levels were increased (3 fold) and NOX (2 fold).	The mean value of TUNEL-positive cells in the hippocampal CA1 sections was elevated (8 fold) and the activation of caspase-8 and caspase-3 was increased.	Wu et al., 2012		Same dose	Incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction)	
DomA	Mouse cerebellar granule neurons (CGNs) from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		Time course (15 to 120 min)	DomA caused a significant time- and concentration-dependent increase in ROS production. The higher ROS production (2.5 fold increase) was recorded after 1 h of exposure.	IC50 values for DomA were 3.4 µM in Gclm (+/+) neurons and 0.39 µM in Gclm (-/-) neurons based on MTT assay after 24 h of exposure.	Giordano et al., 2006	KE up (mitochondrial dysfunction) happens earlier than KE down (cell death)	Same doses		

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Mitochondrial dysfunction (KE up)	Cell death (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	CGNs from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		Time course (0 to 180 min)	DomA (0.1µM) caused a 3 fold increase in DHR fluorescence, which accumulates in mitochondria and fluoresces when oxidized by ROS or reactive nitrogen species. This occurred between 1 and 2 h and was higher in CGNs from Gclm (−/−) mice.	0.1µM DomA was maximally effective in inducing apoptosis, while a concentration causing high toxicity (10µM) induced very limited apoptosis, 24 h after exposure.	Giordano et al., 2007	KE up (mitochondrial dysfunction) happens earlier (1-2 h) than KE down (cell death) that occurs after 24 h	Same doses		
DomA	CGNs from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		For ROS: 30min, Apoptosis: 12-24 h.	ROS levels were measured only at the dose of 100 nM DomA 30 min after treatment in CGNs from Gclm (+/+) mice and showed 2.5 fold increase compared to controls .	A dose response study that showed that even 50 nM DomA exposure for 1 h (after washout and additional 23 h incubation) can induce apoptosis in CGNs from Gclm (+/+) mice, whereas neurons from Gclm (−/−) mice that have very low levels of glutathione were more sensitive as 10 nM DomA induced a significant increase in apoptotic cells number .The maximal apoptosis (5 fold compared to	Giordano et al., 2009	KE up (mitochondrial dysfunction) happens earlier (30 min) than KE down (cell death) that take place 12-24 h later	Same dose	Incidence of downstream KE (cell death) is higher than the incidence of upstream KE (mitochondrial dysfunction)	

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Mitochondrial dysfunction (KE up)	Cell death (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
						controls) in CGNs from both genotypes was caused by 100 nM DomA. 1 and 10 µM DA caused significant apoptosis in both cell types but to less extend compared to 100 nM DomA. Caspase 3 activity after 12 h with prior 1 h exposure to 100 nM DomA found to be increased (2.2 fold). DomA (100 nM) caused a significant decrease (25%) of Bcl-2 protein levels after 6 h from exposure.					

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Mitochondrial dysfunction (KE up)	Cell death (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Mixed cortical cultures obtained from pregnant Holtzman rats on embryonic day (ED) 16–18	3, 5, 10, or 50 μM		10 min, 30 min, 1 h or 2 h, after which DomA was washed out and the culture medium replaced with conditioned medium from unexposed sister cultures .		EC50 exposure paradigms have been established, which represent weak/prolonged exposure (3 μM/24 h), moderate concentration and duration exposure (10 μM/2 h), and strong/brief exposure (50 μM/10 min) .	Qiu et al., 2006				
DomA	Rat	1 mg/kg DA	i.p.		Indirectly it has been shown that ROS production is associated with these histopathological findings by using the radical scavenger edaravone .	Neuronal shrinkage and cell drop out as well as increase in the percentage of TUNEL positive cells at 24 hours (8.3 %) and 5 days (19.0 %) has been found compared with that of controls (1.7 %) .	Tsunekawa et al., 2013				

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Mitochondrial dysfunction (KE up)	Cell death (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Rat rain slices from 8-day-old pups	10 µM		Time course (12, 24, 48 and 92 h) after DomA treatment.		PI uptake (DomA/control) was 14.5 and 34.5 in cortex and hippocampus, respectively . Degradation of α-spectrin to the 120-kDa product after 18 h of DomA treatment was noted but no change was noted after 12 h incubation, whereas caspase 3 activity results were not conclusive.	Erin and Billingsley, 2004				
DomA	Cultured murine cortical neurones					DomA induces concentration-dependent neuronal cell death and the EC50 determined to be 75 µM .	Larm et al., 1997				

Gap of knowledge: there are no studies showing that GLF induces neuronal cell death through mitochondrial dysfunction.

Uncertainties or Inconsistencies

Rats have been administered with DA at the dose of 1.0 mg/kg for 15 days. The histochemical analysis of hippocampus from these animals has revealed no presence of apoptotic bodies and no Fluoro-Jade B positive cells (Schwarz et al., 2014).

Quantitative Understanding of the Linkage

The experiments describing semi-quantitative effects for this KER is described in the table above.

Evidence Supporting Taxonomic Applicability

Neuronal necrosis has been noted in sea lions accidentally exposed to DomA (Silvagni et al., 2005) that correlated well with the histopathological findings previously reported in experimental studies (Tryphonas et al., 1990).

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5. Cell death, N/A leads to Neurodegeneration, N/A

How Does This Key Event Relationship Work

Weight of Evidence

Biological Plausibility

There is well established mechanistic understanding supporting the relationship between these two KEs.
Neurodegeneration in the strict sense of the word is referring to any pathological condition primarily affecting brain cell populations (Przedborski et al., 2003). At histopathological level, neurodegenerative conditions are described by neuronal death and reactive gliosis (Przedborski et al., 2003).

Empirical support for linkage

Domoic acid (DomA)

- Acute brain damage induced by DomA is characterised by neurodegenerative changes consisting of neuronal shrinkage, vacuolization of the cytoplasm, cell drop out, edema, microvacuolation of the neuropil and hydropic cytoplasmic swelling of resident astrocytes (reviewed in Pulido, 2008). These histopathological changes can be identified within structures of the limbic system, in hippocampus, in the CA3, CA4 or hilus of the dentate gyrus (DG) (reviewed in Pulido, 2008). Other brain areas known to be affected by DomA include: the olfactory bulb, the piriform and entorhinal cortices, the lateral septum, the subiculum, the arcuate nucleus and several amygdaloid nuclei. The area postrema is another target for DomA toxicity as it has been identified in both rodents and non-human primates, providing a possible explanation of emetic symptoms (nausea, retching, and/or vomiting) induced by DomA. There has been an effort to map and create a 3-D reconstruction of DomA-induced neurodegeneration in the mouse brain demonstrating that the affected areas include the olfactory bulb, septal areas and the limbic system (Colman et al., 2005; Barlow et al., 2004).

- Female Sprague-Dawley rats dosed once intraperitoneally (i.p.) with 0, 1, 2, 4, or 7.5 DomA mg /kg of body weight have been euthanized after 24 h and their nervous system has been examined for microscopic alterations revealing neuronal degeneration and vacuolation of the neurophil in the limbic and the olfactory systems (Tryphonas et al., 1990).

- The mean of TUNEL positive cells in the hippocampus is increased (6 fold) in mice injected intraperitoneally (i.p.) at a dose of 2 DomA mg/kg once a day for 3 weeks (Lu et al., 2012). However, the same treatment protocol does not cause any neurodegeneration (Lu et al., 2012). In contrast, when the same treatment has been prolonged for one more week (total 4 weeks), the mean values of NeuN-positive cells in the hippocampal CA1 sections of DomA-treated cells decreases by 3 fold compared to controls (Lu et al., 2012). This study shows that the incidence of upstream KE (cell death) is higher than the incidence of downstream KE (neurodegeneration) and that upstream KE (cell death) precedes downstream KE (neurodegeneration).

- The bcl-2 and bax mRNA levels in the hippocampus are significantly increased at 16 h and gradually decreased at 24 h following the administration of DomA (0.75 mg/kg body weight) in adult rats. In situ hybridization analysis reveals complete loss of bcl-2, bax, and caspase-3 mRNA at 24 h after DA administration in the region of the hippocampus, whereas neurodegeneration by Nissl staining is detected at the same time point but has been reported to be more pronounced after 5 days (Ananth et al., 2001). This study demonstrates that both KEs occur after exposure to the same dose of DomA and that the upstream KE (cell death) occurs earlier than the downstream KE (neurodegeneration).

- Adult rats received i.p. injections with DomA 1.0 mg/kg/h until animals exhibited first motor seizures. After a week of recovery, aggressive behaviors and motor seizures of the animals have been monitored for 3h twice a week. After 12 weeks, animals were euthanised and brains have been examined for indications of cell loss by using thionine (Nissl) staining, which highlights the cell bodies of all living neurons. In piriform cortex a reduced cell density has been noted in the medial layer 3 (1.3-1.8 fold decrease compared to controls), an area that shows also prominent amino cupric staining (stain that assesses

neuronal damage) (Tiedeken and Ramsdell, 2013a). The same research group has reported that by following the above experimental procedure but sacrificing the rats 7 days after DomA-induced seizures intense and widespread silver reaction product in the olfactory bulb occurs, whereas minor or no evident damage is found in the hippocampus (Tiedeken et al., 2013b).

- Injection of DomA 0.5 mg/kg, i.p. to adult C57BL/6 male mice results in loss of 32% and 30% of Nissl-stained neurons in hilus and CA1 pyramidal layer of the hippocampus, respectively, compared to control mice when they are sacrificed 7 d after the administration (Antequera et al., 2012).

- The severity and extent of hippocampal neuronal degeneration varies significantly depending on the dose of DomA (1 μM to 1 mM) that is tested after microinjection to adult male Sprague Dawley rats (Qiu and Currás-Collazo, 2006). In rats dosed with 1 mM DomA and sacrificed after 24 h, histopathological analysis using toluidine blue staining has revealed extensive neuronal damage throughout the ipsilateral hippocampal structure. Shrunken, disorganized and densely stained neurons of irregular shape have been identified throughout CA1, CA2, CA3 pyramidal layer as well as the dentate gyrus hilus and granule cells layer. For the 100 μM group animals, CA1 neuronal changes have been less prominent, whereas 10 μM and 1 μM DomA have not produced any resolvable histopathological changes (Qiu and Currás-Collazo, 2006).

- Adult male rats treated with 2 mg/kg DomA i.p. have been sacrificed after 3 d and showed that the silver stain that is used to assess neurodegeneration clearly distinguishes treated from control animals, whereas a number of other markers has failed to do so (Scallet et al., 2005). The same results have been found after even longer exposure times (7 d) to DomA (Appel et al., 1997).

- Male Wistar rats have been given a single i.v. injection of DA (0.75 mg/kg) in the right external jugular vein and brain sections have been stained with Nissl stain at 5 d after DomA administration. Histopathological analysis has revealed a large number of darkly stained shrunken neurons in the hippocampus (Ananth et al., 2003). However, complete absence of hippocampal neurons has been observed in CA1 and CA3 regions in DomA treated animals at 3 months after DomA administration (Ananth et al., 2003).

- In 2-3 week old hippocampal slice cultures, derived from 7 day old rat pups, DomA (0.1-100 μM) has been added to the culture medium and neurodegeneration in the fascia dentata (FD), CA3 and CA1 hippocampal subfields has been measured. The CA1 region appears to be most sensitive to DomA, with an EC50 value of 6 μM DomA after estimating the PI-uptake at 72 h (Jakobsen et al., 2002).

- Cynomolgus monkeys have been given i.v. a range of DomA doses from 0.25 to 4.0 mg/kg. Silver staining of brain sections have revealed that doses in the range of 0.5-1.0 mg/kg produces a small area of silver grains restricted to axons of the hippocampal CA2 stratum lucidum, whereas higher concentrations produce degenerating axons and cell bodies (Slikker et al., 1998). The same research group treated (i.v.) adult monkeys with DomA at one of a range of doses from 0.25 to 4 mg/kg. After a week, silver staining has

demonstrated degenerating axons and cell bodies that are restricted to CA2 stratum lucidum at a lower doses (0.5 to 1.0 DomA mg/kg). Doses of more than 1.0 mg/kg cause widespread damage to pyramidal neurons and axon terminals of CA4, CA3, CA2, CA1, and subiculum subfields of the hippocampus. However, when DomA is orally administered to cynomolgus monkeys at doses of 0.5 mg/kg for 15 days and then at 0.75 mg/kg for another 15 days no histopathoogical changes in the brain are detected (Truelove et al., 1997).

- In humans, autopsy of individuals intoxicated by DomA reveal brain damage characterised by neuronal necrosis and in the hippocampus and the amygdaloid nucleus (Pulido, 2008). The thalamus and subfrontal cortex are damaged only in some patients suffering from Amnesic Shellfish Poisoning (ASP). The detailed examination of one patient intoxicated by DomA has revealed complete neuronal loss in the CA1, CA3 and CA4 regions, whereas moderate loss is seen in the CA2 region (Cendes et al., 1995). Non-severe neuronal loss has been detected in amygdale, overlying cortex, the dorsal and ventral septal nuclei, the secondary olfactory areas, and the nucleus accumbens (Cendes et al., 1995).

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Cell death (KE up)	Neurodegeneration (KE down)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Female Sprague-Dawley rats	0, 1, 2, 4, or 7.5 DomA mg /kg	Intraperitoneally (i.p.)	Euthanised after 24 h		Neuronal degeneration and vacuolation of the neuropil in the limbic and the olfactory systems	Tryphonas et al., 1990				
DomA	16-month-old male ICR mice	2 mg/kg	i.p.	Once a day for 3 or 4 weeks	The mean of TUNEL positive cells in the hippocampus was increased (6 fold). The levels of bcl-2, procaspase-3 and procaspase-12 were significantly decreased and the activation of caspase-3 and caspase-12 in the mouse hippocampus were increased.	The mean OD of NeuN immunoreactivity in the hippocampus of mice decreased (3 fold) indicating significant neuron loss by apoptosis, which is one of the pathological hallmarks of neurodegeneration	Lu et al., 2012	Upstream KE (cell death) precedes downstream KE (neurodegeneration)	Same dose	Incidence of upstream KE (cell death) is higher than the incidence of downsteam KE (neurodegeneration)	Mice treated with DomA once a day for 3 weeks showed that apoptosis was increased. However, the same treatment protocol did not cause any neurodegeneration. In contrast, when the same treatment has been prolonged for one more week (total 4 weeks) induced marked neuron loss.

DomA	Adult rats	0.75 mg/kg	intravenously (i.v.)	Euthanized after 2, 5, 14, or 21 days	The bcl-2 and bax mRNA levels in the hippocampus were significantly increased at 16 h and gradually decreased at 24 h following the administration of DomA. In situ hybridization analysis revealed complete loss of bcl-2, bax, and caspase-3 mRNA at 24 h after DomA administration in the region of hippocampus	Neurodegeneration by Nissl staining was detected at the same time point but was reported to be more pronounced after 5 days	Ananth et al., 2001	Upstream KE (cell death) occurs earlier that downstream KE (neurodegeneration).	Same dose		
DomA	Adult rats	1.0 mg/kg/h until animals exhibited first motor seizures	i.p.	Euthanized after 12 weeks		In piriform cortex a reduced cell density was noted in the medial layer 3 (1.3-1.8 fold decrease compared to controls), an area that showed also prominent amino cupric staining (stain that assesses neuronal damage).	Tiedeken and Ramsdell, 2013a				

DomA	Adult rats	1.0 mg/kg/h until animals exhibited first motor seizures	i.p.	Euthanized after 1 week		Intense and widespread silver reaction product in the olfactory bulb, whereas minor or no evident damage was found in hippocampus.	Tiedeken et al., 2013b				
DomA	Adult C57BL/6 male mice	0.5 mg/kg	i.p.	Euthanized after 1 week		DomA treatment resulted in the loss of 32% and 30% of Nissl-stained neurons in hilus and CA1 pyramidal layer of the hippocampus, respectively, compared to control mice.	Antequera et al., 2012				

DomA	Adult male Sprague Dawley rats	1 μM to 1 mM	microinjection	Euthanized after 24 h		In rats dosed with 1 mM DomA and sacrificed after 24 h, histopathological analysis using toluidine blue staining revealed extensive neuronal damage throughout the ipsilateral hippocampal structure. Shrunken, disorganized and densely stained neurons of irregular shape were identified throughout CA1, CA2, CA3 pyramidal layer as well as the dentate gyrus hilus and granule cells layer. For the 100 μM group animals, CA1 neuronal changes were less prominent, whereas 10 μM and 1 μM DomA did not produce resolvable histopathological changes.	Qiu and Currás-Collazo, 2006				

DomA	Adult male rats	2 mg/kg	i.p.	Euthanized after 3 or 7 days		DA treatment for 3 d showed that the silver stain that was used to assess neurodegeneration clearly distinguished treated from control animals, the same was true for longer exposure time (7 d).	Scallet et al., 2005, Appel et al., 1997				
DomA	Male Wistar rats	0.75 mg/kg	i.v.	Euthanized after 5 days or 3 months		Histopathological analysis revealed a large number of darkly stained shrunken neurons in the hippocampus However, complete absence of hippocampal neurons were observed in CA1 and CA3 regions in DA treated animals at 3 months after DomA administration.	Ananth et al., 2003				

DomA	2-3 week old rat hippocampal slice cultures, derived from 7 day old rat pups	0.1-100 µM		72 h		DomA induced neurodegeneration in the fascia dentata (FD), CA3 and CA1 hippocampal subfields. The CA1 region appeared to be most sensitive to DomA, with an EC50 value of 6 µM DomA, estimated from the PI-uptake at 72 h .	Jakobsen et al., 2002				

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

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

Uncertainties or Inconsistencies

Zebrafish has been exposed for 36-weeks to DomA and has shown no excitotoxic neuronal death and no histopathological lesions in glutamate-rich brain areas (Hiolski et al., 2014).
Administration of DomA (9.0 mg DomA kg(-1) bw, i.p.) to Sparus aurata (seabream) leads to measurement of 0.61, 0.96, and 0.36 mg DomA kg(-1) of brain tissue at 1, 2 and 4 hours. At this dose but also at lower concentrations (0.45 and 0.9 mg DomA kg(-1) bw) no major permanent brain damage has been detected (Nogueira et al., 2010). Leopard sharks possess the molecular target for DomA but it has been shown to be resistant to doses of DomA that can cause neurotoxicity to other vertebrates, suggesting the presence of some protective mechanism (Schaffer et al., 2006).
All these reports support the view that there is possible a species specific susceptibility to DomA toxicity.

Quantitative Understanding of the Linkage

The experiments describing semi quantitative effects for this KER are provided in the table above.

Evidence Supporting Taxonomic Applicability

There is an overall agreement regarding the histopathology of the brain lesions related to acute DomA neurotoxicity across certain species. Data derived from humans, rodents, non-human primates and sea lions suggest that common neudegeneration features in selected brain areas are found despite the fact that study design, estimated exposure, processing of samples and history of event may differ (Pulido, 2008).
Furthermore, the distribution of brain damage by DomA has also been established by magnetic resonance imaging microscopy (MRM) for both human and rat, demonstrating similar distribution as that described by histopathological studies (Pulido, 2008).
It is important to notice that human sensitivity to DomA exposure is well documented in the published literature and seems to be much higher than in other species (Lefebvre and Robertson 210; Barlow et al., 2004). In 1987 in Canada, more than 200 people became acutely ill after ingesting of mussels contaminated with DomA. The outbreak resulted in 20 hospitalisations and four deaths. Clinical effects observed included gastrointestinal symptoms and neurotoxic effects such as hallucinations, memory loss and coma. For this reason, the condition was termed amnesic shellfish poisoning (Barlow et al., 2004). The neurotoxic properties of domoic acid result in neuronal degeneration and necrosis in specific regions of the hippocampus (Teitelbaum et al., 1990).

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6. Cell death, N/A leads to Neuroinflammation, N/A

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; Ransohoff and Brown, 2014). Toll-like receptors (TLRs) are pattern-recognition receptors that recognise 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 leads to neuroinflammation (microglial and astrocyte reactivities) is widely accepted.

Empirical support for linkage

Domoic acid (DomA)

- 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-1β (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).

Pb^{2+}

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 young mice (Sobin et al., 2013) as well as 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-1β, 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 impaiment of glutamate uptake and probable neuronal injury (Struzunska, 2000; Struzunska et al., 2001).

Domoic acid (DomA)
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).

Pb^{2+}
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). A possible way to explain this inconsistency is 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 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).

Quantitative Understanding of the Linkage

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

Evidence Supporting Taxonomic Applicability

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).

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7. Neurodegeneration, N/A leads to Neuroinflammation, N/A

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 pathogeny 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. Microglial 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

Domoic acid (DomA)

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.

Uncertainties or Inconsistencies

Quantitative Understanding of the Linkage

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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8. Neuroinflammation, N/A leads to Neurodegeneration, N/A

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 and Bal-Price, 2003; Kraft and Harry, 2011; Taetzsch & 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 chronical 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

Domoic acid (DomA)

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).

Pb^{2+}

Rats treated during pregnancy from gestational day 5 throughout life till postnatal day 180 with a mixture of Pb/Cd/As showed in early adulthood increased levels of IL-1β, IL-6 and TNF-α in hippocampus and frontal cortex associated with increased Aβ levels (Ashok et al., 2015). Similarly, monkeys exposed during infancy to Pb (1 mg/kg/day from birth to 400 days) showed in aging (23 y old) an overexpression of APP and Abeta (Bihaqi et al., 2011), and of Tau mRNA and protein (Bihaqi and Zawa, 2013). Similar observations were made in old rats (18-20 months) after early life exposure to Pb (0.2% in drinking water from postnatal day 1 to 20) (Basha et al., 2005; Zawia and Basha, 2005; Bihaqi et al., 2014). This was associated with cognitive impairment that was observed only if animals were exposed when young (Bihaqi et al., 2014). 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 peristent brain damage measured by MRI (Stewart et al., 2006). Some in vitro experiments also show that neuroinflammation can lead to degeneration: the conditionned medium of Pb-treated microglial cells (10 microM for 12h) caused the death of neuroblastoma cells (Kumawak et al., 2014). And 3D cultures treated with Pb for 10 days exhibited neuroinflammation accompanied by neuronal death (Zurich et al., 2002).

Uncertainties or Inconsistencies

Long-term treatments with NSAIDs (non-steroidal anti-inflammatory drugs) have a preventive effect on Alzheimer's disease development (Piertrzick 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 postanatally (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

There are no model and no dose-response experiments allowing to link neuroinflammation and neurodegeneration. The development of neurodegeneration may not depend on the quantity/intensity of neuroinflammation, but rather on the properties of the neuroinflammatory process (e.g. chronicity, expression of the neurodegenerative M1 phenotype by microglial cells,...)

Evidence Supporting Taxonomic Applicability

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

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9. Neurodegeneration, N/A leads to Neuronal network function in adult brain, Decreased

How Does This Key Event Relationship Work

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

Weight of Evidence

Biological Plausibility

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

Empirical support for linkage

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

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

prolonged application of 400 nM DA reversibly depresses CA1 fEPSP and impairs the subsequent development of tetanus LTP (Qiu et al., 2009).

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

Uncertainties or Inconsistencies

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

Quantitative Understanding of the Linkage

Not enough information exists to understand this linkage quantitativly.

Evidence Supporting Taxonomic Applicability

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

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10. Neuronal network function in adult brain, Decreased leads to Learning and memory, Impairment

How Does This Key Event Relationship Work

It is well established in the existing literature that NMDA receptor–dependent synaptic potentiation (LTP) and depression (LTD) are two forms of activity directly linked to long-term changes in synaptic efficacy and plasticity, the fundamental processes underlying learning and memory. The best characterised form of LTP occurs in the CA3-CA1 region of the hippocampus, in which LTP is initiated by transient activation of NMDARs that leads to a persistent increase in synaptic transmission through AMPA receptors (Benke et al., 1998) that can be achieved either through increasing the number of AMPA receptors at the post-synaptic surface or by increasing the single channel conductance of the receptors expressed. It has been shown that LTP in the CA1 region of the hippocampus could be accounted for by these two mechanisms (Benke et al 1998). The degree of activity of NMDARs is determined in part by extracellular Mg(2+) and by the co-agonists for this receptor, glycine and D-serine. During strong stimulation, a relief of the voltage-dependent block of NMDARs by Mg(2+) provides a positive feedback for NMDAR Ca(2+) influx into postsynaptic CA1 spines. The induction of LTP at CA3-CA1 synapses requires further signal amplification of NMDAR activity. Src family kinases (SFKs) play a "core" role in the induction of LTP by enhancing the function and expression of NMDARs. 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. Many transmitters and signal pathways can modify NMDAR function and expression and, for a given stimulus strength, they can potentially lead to a change in the balance between LTP and LTD (MacDonald et al., 2006).

Mammalian learning and memory is one of the outcomes of the functional expression of neurons connected into neural networks. Neuronal damage or cell death induced by chemical compounds disrupts integration and transmission of information through neural networks thereby setting the stage for subsequent impairment of learning and memory. Exposure to chemicals that will increase the risk of functional neuronal network damage lead to learning and memory impairment.

Weight of Evidence

Biological Plausibility

Long-term potentiation (LTP) is a long-lasting increase in synaptic efficacy after high-frequency stimulation of afferent fibers, and its discovery potentiated the idea that individual synapses possess the properties expected for learning and memory (reviewed in Lynch et al., 2014). Moreover, LTP is intimately related to the theta rhythm, an oscillation long-associated with learning. Learning-induced enhancement in neuronal excitability, a measurement of neural network function, has also been shown in hippocampal neurons following classical conditioning in several experimental approaches (reviewed in Saar and Barkai, 2003). On the other hand, memory requires the increase in magnitude of EPSCs to be developed quickly and to be persistent for a at least a few weeks without disturbing already potentiated contacts. Once again, a substantial body of evidence has demonstrated that tight connection between LTP and diverse instances of memory exist (reviewed in Lynch et al., 2014).

The recent studies suggest that NMDA receptor-dependent long-term depression of both LTD and LTP is usually accompanied by morphological changes in spines. LTD is characterised by long lasting dendritic spine shrinkage and reduced F-actin polymerization, in addition to reduced numbers of synaptic AMPA receptors. Moreover, the actin binding protein cofilin has been implicated in mediating such synaptic structural plasticity (Chen et al., 2007). If sustained, such LTD-changes in hippocampus or cortex, triggered by NMDARs overactivation could lead to synaptic dysfunction, contributing to learning and memory damage (Calabrese et al., 2014).

Empirical support for linkage

A series of important findings support that the biochemical changes that happen after induction of LTP also occur during memory acquisition, showing temporality between the two KEs (reviewed in Lynch et al., 2014). Furthermore, a review of Morris water maze (MWM) data as a tool to investigate spatial learning and memory in laboratory rats also pointed to the disconnection between neuronal networks rather than brain damage of certain regions is responsible for the impairment of MWM performance(D'Hooge and De Deyn, 2001). Functional integrated neural networks that involve the coordination action of different brain regions are consequently important for spatial learning and MWM performance. Morris et al. 1986 found that blocking the NMDA receptor with AP5 inhibits spatial learning in rats. More importantly, in the same study they measured brain electrical activity and recorded that this agent also inhibits LTP, however, they did not prove that spatial learning and LTP inhibition are causally related (Morris et al., 1986). Since then a number of NMDA receptor antagonists have been studied for their ability to induce impairment of learning and memory. It is worth mentioning that similar findings have been found in human subjects, where by combining behavioural and electrophysiological data from patients with temporal lobe epilepsy exposed to ketamine, the involvement of NMDA receptors in human memory processes was demonstrated (Grunwald et al., 1999).

Domoic Acid (DomA)

Mice exposed to DomA (2.0 mg/kg) showed impairment of the acquisition of the place task in the Morris water maze (Petrie et al., 1992). These animals also failed to select the appropriate problem-solving strategies in their attempt to search for the underwater platform (longer escape latencies than controls) (Petrie et al., 1992). This impairment of acquisition and retention of this spatial navigation task by DomA, was associated with neuronal damage not only in the hippocampus, but also in limbic brain regions (Petrie et al., 1992). Similar results were obtained in a different study that also utilised the Morris water maze but in rats (Kuhlmann and Guilarte, 1997). These animals received DomA (1.5 or 3.0 mg/kg i.p.) and exhibited significant learning deficits while animals treated with a lower dose (0.75 mg/kg) showed no deficits. By incorporating in the test a visual cue trial (a black flag was positioned over the goal platform to eliminate the learning and memory components), it was possible to rule out sensory and motor deficits in the treated animals unable to solve the task (Kuhlmann and Guilarte, 1997).

Rats were examined using a neurobehavioral test battery (passive avoidance, auditory startle and conditioned avoidance) after DomA treatment with 0, 0.22, 0.65, or 1.32 mg/kg i.p. that caused hardly any measurable brain injury (Sobotka et al., 1996). Approximately 25% of the animals that received the higher dose (1.32 mg/kg) DomA died or were euthanized. Surviving animals were assessed three days after exposure and showed changes on the auditory startle test (Sobotka et al., 1996). This effect was limited to exaggerated startle responses as measured by mean-response amplitude changes and did not include changes in habituation, suggesting the presence of behavioural hyper-reactivity rather than memory impairment. No change on the other two measures of avoidance learning was detected for all DomA treated groups compared to controls (Sobotka et al., 1996).

In an experimental approach aiming to evaluate single versus repeated doses of DomA on learning and memory, adult mice were treated with single or four i.p. injections of 1.0 or 2.0 mg/kg over a seven day period. All animals were tested on a spatial delayed matching-to-sample test in Morris water maze (Clayton et al., 1999). Animals given a single dose of 2.0 mg/kg DomA performed more poorly than controls on “nonalternation” test days; sessions in which the correct response was the same as the preceding day (Clayton et al., 1999). This finding implies that DomA-treated animals had difficulty recalling information after a 24 h delay period and were exhibiting behavioural signs similar to human antegrade amnesia. Animals exposed to multiple doses displayed initially greater general symptomatology but after recovery, did not show greater cognitive impairment than subjects treated with a single injection (Clayton et al., 1999).

In the only study where DomA i.v. administration was tested in relation to impairment of learning and memory, rats were dosed with 0.04 μg DomA/kg (and diazepam, ip). Radial arm maze tests revealed severe learning impairment, suggesting deficits in working memory. In subjects that were able to ultimately solve the maze, performance deficits were documented on re-learning the same task. The memory-based deficits observed in these animals are similar to human antegrade amnesia that has been reported after DomA exposure (Nakajima and Potvin, 1992). In humans, the hallmark of DomA-induced neurotoxicity is the rather dramatic disruption of memory processing. Clinical evaluation of 14 adult human subjects poisoned by DomA (after the outbreak in Canada) revealed that the majority of them (12/14) had severe antegrade memory deficits with relative preservation of higher cortical functions, since the patients were unable to remember

events that occurred after DomA intoxication and had difficulty recalling new information (reviewed in Pulido, 2008; Grant et al., 2010).

In a separate clinical report of one case, a patient that received treatment at the hospital for DomA poisoning showed memory impairment which was resolved after three weeks (reviewed in Pulido, 2008; Grant et al., 2010). Other symptoms of the syndrome include nausea, vomiting, abdominal cramps, diarrhoea, headache, unstable blood pressure, cardiac arrhythmias and neurological dysfunction, including coma and seizures (reviewed in Pulido, 2008; Grant et al., 2010).

Treatment of female Sprague-Dawley rats with 0, 1, 2, 4, or 7.5 mg domoic acid/kg of body weight for 24 h caused neurobehavioural changes at doses above 4 mg/kg of body weight characterised by unequivocal behavioural and neurological signs leading to partial seizures and status epilepticus (Tryphonas et al., 1990). Similar effects were observed in other in vivio studies (Fuquay et al., 2012; Muha and Ramsdell, 2011).

The exposed zebrafish from the 36-week treatment with DomA showed no visible signs of neurobehavioral excitotoxicity (i.e.circle- or spiral-swimming) when observed over a 30–45 min period following each injection (Hiolski et al., 2014). The latencies measured during training sessions did not differ among treated and not treated animals but the step-through latency in the 24 h retention trial was significantly lowered in DomA-exposed mice (2 mg/kg once a day for 3 weeks) (Lu et al., 2012). DomA-treated mice had longer escape latencies compared to controls in Morris water maze. The same group reported that DomA-treated mice (2 mg/kg once a day for 3 weeks) compared to controls showed a decrease (4.5 fold) in the step-through latency in the 24 h retention trial, a decrease (4 fold) in the number of crossings over the exact former location of the platform and a reduction (3 fold) in the time spent in the target quadrant (where the platform was located during hidden platform training) during the MWM task probe test (Wu et al., 2013, Wu et al, 2012). In this study FoxO1 knockdown reversed the cognitive deficits induced by DA in mice (Wu et al., 2013).

Adult male and female Sprague Dawley rats received a single intraperitoneal injection of DomA (0, 1.0, 1.8 mg/kg). These low levels of DomA showed that males may be more susceptible to severe neurotoxicity, whereas females are affected more quickly as it increased locomotor and grooming activity after monitoring behaviour for 3h (Baron et al., 2013).

Glufosinate (GLF)

1. **GLF impairs neuronal network function**. The networks of cortical rat neurons were exposed to glufosinate (GLF) and its primary metabolite N-acetylglufosinate (NAcGLF) and electrical activity was measured using microelectrode array (MEA). The MEA recordings showed the concentration-responses for GLF and NAcGLF on network mean firing rates (MFR) that were biphasic, increasing at lower concentrations, decreasing below control levels at higher concentrations (similarly to NMDA). Increases in MFR occurred between 100–300 uM for NAcGLF (190% control, maximum) and 10–1000 uM for GLF (340% control, maximum) indicating that GLF is affecting neuronal network function (Lantz et al., 2014). Toxicokinetic data from reports of intentional human poisonings indicate that GLF concentrations in the CNS could reach levels high enough to lead to effects mediated via NMDARs (Watanabe and Sano, 1998).
Direct activation of NMDARs by GLF is also suggested by in vivo studies where three NMDA receptor antagonists, dizocilpine, LY235959, and Compound 40, and AMPA/KA antagonist,

NBQX, were co-administrated with glufosinate ammonium (80 mg/kg, intraperitoneally) in mice. Statistical analyses showed that the NMDA receptor antagonists markedly inhibited the GLF-induced convulsions, while the AMPA/KA receptor antagonist had no effect. These results suggest that the convulsion caused by glufosinate ammonium were mediated through NMDA receptors (Matsumura et al., 2001).

2. **GLF exposure triggers convulsions and memory impairment.** GLF expsoure produces moderate to severe convulsions and memory loss (Koyama et al., 1994; Watanabe and Sano, 1998; Ohtake et al., 2001; Park et al., 2006, 2013; Mao et al., 2011a and b, 2012), as well as causes structural changes to several brain regions, including the cortex and hippocampus (Calas et al., 2008; Meme et al., 2009; Park et al., 2006), two brain structures rich in NMDARs that play an important role in learning and memory processes.

• A 64 year old patient who ingested GLF suffered mental disturbances and later developed generalized convulsions, impaired respiration and circulatory failure. During recovery he exhibited loss of short-term memory (retrograde and anterograde amnesia) (Watanabe and Sano, 1998).

• Similarly, a 34-yr-old man who ingested glufosinate ammonium developed mental deterioration (Park et al., 2013).

• Retrograde amnesia has been reported following acute GLF toxicity in humans (Park et al., 2006).

• Chronic GLA (glufosinate-ammonium) treatments with 5 and 10mg/kg induce mild memory impairments and a modification of hippocampal texture in mice. It is suggested that these modifications may be causally linked one to another. Hippocampal MRI texture and spatial memory alterations might be the consequences of hippocampal glutamate homeostasis modification (Calas et al., 2008).

• Spatial memory impairment was observed in mice after chronic exposure to as low as 2.5 mg/kg of GLF (Calas et al., 2008; Meme et al., 2009).

Uncertainties or Inconsistencies

One of the most difficult issues for neuroscientists is to link neuronal network function to cognition, including learning and memory. It is still unclear exactly what modifications in neuronal circuits need to happen in order to alter motor behaviour as it is recorded in a learning and memory test (Mayford et al., 2012), meaning that there is no clear understanding about how these two KEs are connected.

It is unclear whether GLF affects only glutamatergic systems since other potential mechanisms underlying GLF neurotoxicity have not been widely investigated. Based on the existing data it is understood that exposure to GLF or NAcGLF could disrupt the neuronal network function through disruption of glutamatergic neurotransmission but further work is required to clarify molecular mechanisms that cause impairment of memory.

Quantitative Understanding of the Linkage

Quantitative evaluation of this KER does not exist.

Evidence Supporting Taxonomic Applicability

Name	Scientific Name	Evidence	Links
human	Homo sapiens	Strong	NCBI
mouse	Mus sp.	Strong	NCBI
rat	Rattus sp. ABTC 42503	Strong	NCBI

Administration of DomA (9.0 mg DomA kg(-1) bw, i.p.) to Sparus aurata (seabream) caused neurological disturbances such as swimming in a circle, in a spiral, or upside down, that were reversed 24 hours after exposure (Nogueira et al., 2010). In rainbow trout (Oncorhynchus mykiss), DomA (0.75 mg/kg bw) administration caused increased aggressive behaviour 30 min after exposure compared to controls (Bakke et al., 2010).

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Overall Assessment of the AOP

The aim of the present AOP is to construct a linear pathway that captures the KEs and KERs that occur after binding of agonist to NMDA receptor in hippocampal and cortical neurons of adults. The majority of the KEs of the AOP are characterised by MODERATE essentiality for the AO (loss or reduction of cognitive function) or other KEs that follow. The biological plausibility in the majority of KERs is rated STRONG as there is extensive mechanistic understanding. However, the empirical support for the majority of presented KERs cannot be rated high as in most occasions the KEup and KEdown of a KER has not been investigated simultaneously, under the same experimental protocol or not in the suggested brain regions (cortex and hippocampus).

Domain of Applicability

Life Stage Applicability: This AOP is applicable for adults. However, studies exploring the neurotoxic effects of DomA on the developing nervous system demonstrate that DomA can cause neurobehavioral, biochemical and morphological effects similar to the ones observed in adult animals (reviewed in Costa et al., 2010). The DomA doses required to cause these effects in developing organisms are one to two orders of magnitude lower than those needed for loss or reduction of cognitive function in adults. This difference has been attributed to toxicokinetic and/or toxicodynamic particularities that exist between adults and children.

Taxonomic Applicability: The data used to support the KERs in this AOP derives from experimental studies conducted in primates, rats and mice or cell cultures of similar origin as well as from human epidemiological studies or clinical cases of DomA poisoning. The majority of the KEs in this AOP seem to be highly conserved across species. It remains to be proved if these KERs of the present AOP are also applicable for other species rather than human, primates, rats or mice. Increasing evidence from sea lions exposed to DomA further supports some of the KERs of the present AOP.

Sex Applicability: The majority of the studies addressing the KEs and KERs of this AOP have been carried out mainly in male laboratory animals. Few studies are available in females and some

of them compare the effects between females and males. It appears that this AOP is applicable for both females and males.

Essentiality of the Key Events

Molecular Initiating Event	Support for Essentiality
Inotropic glutamate receptors, Binding of agonist	Strong

Key Event	Support for Essentiality
NMDARs, Overactivation	Moderate
Calcium influx, Increased	Moderate
Mitochondrial dysfunction, N/A	Strong
Cell death, N/A	Strong
Neurodegeneration, N/A	Strong
Neuroinflammation, N/A	Weak
Neuronal network function in adult brain, Decreased	Moderate

1) **Essentiality of KE "NMDARs, Overactivation" for the KE "Cell death"** is ***MODERATE***. NMDARs play a central role in excitotoxic neuronal injury. Over-activation of these receptors causes disruption of Ca^{2+} homeostasis that through mitochondrial dysfunction triggers signals leading to apoptotic or necrotic death. However, the ways that cells respond to mitochondrial injury vary and often are considered unclear and controversial (Pivovarova and Andrews, 2010). However, NMDAR antagonists failed to reverse these Ca^{2+} induced cell deaths, leading to suggestions that NMDAR-independent pathways that involve α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), acid-sensing channels and transient receptor potential channels might be also responsible for excitotoxic neuronal injury (Pivovarova and Andrews, 2010). Several agonists have higher affinity than NMDA itself but are not relevant for behavioural studies as NMDA activation leads to epilepsy and cell death, a common approach to induce neurotoxic lesions.

2) **Essentiality of KE "Calcium influx, Increased" for the KE "Cell death"** is ***MODERATE***. Ca^{2+} plays important role in excitotoxicity but the mechanisms involved in excitotoxic cell death are still debated (Berliocchi et al., 2005). Depending on the extent and the duration of the Ca^{2+} influx, neurons survive, die through apoptotic mechanisms in case of sustained slow Ca^{2+} influx, or undergo necrosis when rapid high Ca^{2+} influx occurs. Over-expression of the endogenous calpain inhibitor, calpastatin, or the calpain-resistant isoform the $Na+/Ca^{2+}$ exchanger 2 (NCX2) prevents Ca^{2+} overload and protects neurons from excitotoxicity (Bano et al., 2005).

3) **Essentiality of KE "Mitochondria dysfunction" for the AO "Impairment of learning and memory"** is ***STRONG***. ROS is known to have a negative effect on synaptic plasticity and

learning and memory (reviewed in Lynch, 2004). H_2O_2 inhibits LTP both in vitro and in vivo, which is associated with increased ROS. A negative correlation has been found between ROS concentration in hippocampus and ability of rats to sustain LTP. Administration of antioxidants, vitamins E and C, reverses the inhibitory effects of stress on LTP and prevents the increase of ROS in hippocampus. In transgenic mice that overexpress superoxide dismutase (SOD), the enzyme which catalyzes the conversion of superoxide to H_2O_2, the LTP in CA1 is inhibited. Intracerebroventricular injection of H_2O_2, at a concentration which increases ROS levels in hippocampus, impairs LTP that is prevented after pretreatment with the antioxidant phenylarsine oxide. Knocking down Forkhead box protein O1 (FoxO1) in mice, which is an important regulator of mitochondrial function, reverses mitochondrial abnormalities and cognitive impairment induced by DA in mice (Wu et al., 2013).

4) **Essentiality of KE "Mitochondria dysfunction" for the KE "Cell death"** is ***MODERATE***. There is a considerable number of mitochondrial associated processes that lead to necrotic or apoptotic cell death such as uncoupling of oxidative phosphorylation, activation of the mitochondrial permeability transition pore (MPTP), release of pro-apoptotic proteins, activation of poly(ADP-ribose) polymerase-1 and proteases such as calpain, increased levels of and delayed Ca^{2+} de-regulation (Pivovarova and Andrews, 2010). Although the understanding of these mechanisms is clearly established, the cascade of events and the significance of them are less clear (Pivovarova and Andrews, 2010). A significant body of evidence, both clinical and experimental, supports a role for the mitochondrial permeability transition pore in excitotoxicity (reviewed in Pivovarova and Andrews, 2010). However, the effects of cyclosporin A, the classical MPTP inhibitor, on neuronal mitochondria are inconsistent raising doubts about its role in neural cell death. However, ADP/ATP translocator deficiency, which is not essential for MPTP but does regulate pore opening, protects neurons against excitotoxicity. Furthermore, MPTP opening renders neurons vulnerable to excitotoxicity.

Weight of Evidence Summary

Event	Description	Triggers	Weight of Evidence
Calcium influx, Increased	Indirectly Leads to	Mitochondrial dysfunction, N/A	Strong
NMDARs, Overactivation	Directly Leads to	Calcium influx, Increased	Moderate
Mitochondrial dysfunction, N/A	Indirectly Leads to	Cell death, N/A	Moderate
Cell death, N/A	Directly Leads to	Neurodegeneration, N/A	Moderate
Cell death, N/A	Indirectly Leads to	Neuroinflammation, N/A	Weak
Neuroinflammation, N/A	Directly Leads to	Neurodegeneration, N/A	Moderate

Event	Description	Triggers	Weight of Evidence
Neurodegeneration, N/A	Directly Leads to	Neuroinflammation, N/A	Moderate
Neuronal network function in adult brain, Decreased	Indirectly Leads to	Learning and memory, Impairment	Moderate
Neurodegeneration, N/A	Indirectly Leads to	Neuronal network function in adult brain, Decreased	Weak
Inotropic glutamate receptors, Binding of agonist	Directly Leads to	NMDARs, Overactivation	Strong

The table provides a summary of the biological plausibility and the empirical support for each KER described in this AOP based on "Annex 1: Guidance for assessing relative level of confidence in the overall AOP based on rank ordered elements" found in the User's Handbook.
More information about the evidence that support these KERs and the relevant literature can be found in each KER description.

The main base for the overall scoring is that the empirical support coming from the experiments with one stressor (domoic acid, DomA). However this AOP is not specific for DomA, it is applicable to any chemicals that act as NMDARs agonists.

KERs WoE	Biological plausibility	Does KEup occurs at lower doses than KEdown?	Does KEup occurs at earlier time points than KE down?	Is there higher incidence of KEup than of KEdown?	Inconsistencies/Uncertainties
Binding of agonist to NMDARs directly leads to NMDARs overactivation	Extensive understanding	N/A	Yes	N/A	Limited conficting data
NMDARs overactivation directly leads to increased calcium influx	Extensive understanding	Same dose	Yes	Not investigated	Limited conficting data
Increased calcium influx indirectly leads to mitochondrial dysfunction	Extensive understanding	Same dose	Yes	Yes	No conflicting data
Mitochondrial dysfunction directly leads to cell death	Extensive understanding	Same dose	Yes	Yes	Limited conficting data

Cell death leads to Neurodegeneration	Extensive understanding	Same dose	Yes	Yes	Limited conficting data
Cell death leads to Neuroinflammation	Extensive understanding	Not investigated	Not investigated	Not investigated	N/A
Neurodegeneration directly leads to Decreased neuronal network function	Extensive understanding	Not investigated	Not investigated	Not investigated	N/A
Decreased neuronal network function indirectly leads to loss or reduction of cognitive function	Scientific understanding is not completely established	Not investigated	Not investigated	Not investigated	N/A

Quantitative Considerations

Event	Description	Triggers	Quantitative Understanding
Calcium influx, Increased	Indirectly Leads to	Mitochondrial dysfunction, N/A	
NMDARs, Overactivation	Directly Leads to	Calcium influx, Increased	
Mitochondrial dysfunction, N/A	Indirectly Leads to	Cell death, N/A	
Cell death, N/A	Directly Leads to	Neurodegeneration, N/A	
Cell death, N/A	Indirectly Leads to	Neuroinflammation, N/A	
Neuroinflammation, N/A	Directly Leads to	Neurodegeneration, N/A	
Neurodegeneration, N/A	Directly Leads to	Neuroinflammation, N/A	
Neuronal network function in adult brain, Decreased	Indirectly Leads to	Learning and memory, Impairment	Weak
Neurodegeneration, N/A	Indirectly Leads to	Neuronal network function in adult brain, Decreased	
Inotropic glutamate receptors, Binding of agonist	Directly Leads to	NMDARs, Overactivation	

Considerations for Potential Applications of the AOP

Exposure to xenobiotics can potentially affect the nervous system resulting in neurobehavioral alterations and/or neurological clinical symptoms. To assess the neurotoxic properties of compounds, current testing largely relies on neurobehavioural tests in laboratory animals, histopathological analysis, neurochemical and occasionally electrophysiological observations. Throughout the years, a significant number of methods have been developed to assess neurobehaviour in laboratory animals and a comprehensive summary of them can be found in 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 that are applied to gain information on the neurotoxicity properties of chemical compounds. Namely, these are: general Test Guidelines such as single dose toxicity (e.g. OECD 402, 403, 420, 423 and 425), repeated dose toxicity (e.g. OECD 407 and

408), chronic exposure (e.g. OECD 452) as well as Test Guidelines specifically developed for the study of neurotoxicity in adult laboratory animals, such as OECD Test Guideline for Neurotoxicity (424).

Learning and memory is an important endpoint and a wide variety of tests to assess chemical effects on cognitive functions is available and used for the study of neurotoxicity. Some of these tests that allow the appreciation of cognitive function in laboratory animals are: habituation, ethologically based anxiety tests (elevated plus maze test, black and white box test, social interaction test), conditioned taste aversion (CTA), active avoidance, passive avoidance, spatial mazes (Morris water maze, Biel water maze, T-maze), conditional discrimination (simple discrimination, matching to sample), delayed discrimination (delayed matching-to-sample, delayed alternation) and eye-blink conditioning.

The present AOP can potentially provide the basis for development of a mechanistically informed IATA for neurotoxicity. The construction of IATA for predicting neurotoxic effects in adults is expected to make use of more than one AOP within an interconnected network in order to take into consideration all critical biological processes that may contribute to impairment of learning and memory in adults. Through this network, identification of KEs and KERs common across multiple AOPs can emerge that should be considered during IATA construction and that may inform also in vitro assay development. The development of alternative assays would allow screening of chemicals for potential NMDAR activators and reducing the use of in vivo studies.
Results from assays based on the KEs of this AOP can serve to interpret and accept results that derive from non-standard test methods. Omics data from toxicogenomic, transcriptomic, proteomic, and metabolomic studies can be interpreted in a structured way using this AOP that is relevant to adult neurotoxicity. Currently learning and memory testing is not required by the OECD TG 424. This AOP could serve as a base for chemical evaluation with potential to cause impairment of learning and memory. The assay development would refer to the identified in this AOP KEs that could form a testing strategy for identifying chemicals with potential to cause cognitive deficit. Finally, this AOP could provide the opportunity to group chemicals using not only chemical properties but also mechanistic information that can later inform data gap filling by read-across and predict neurotoxic properties of a target substance.

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