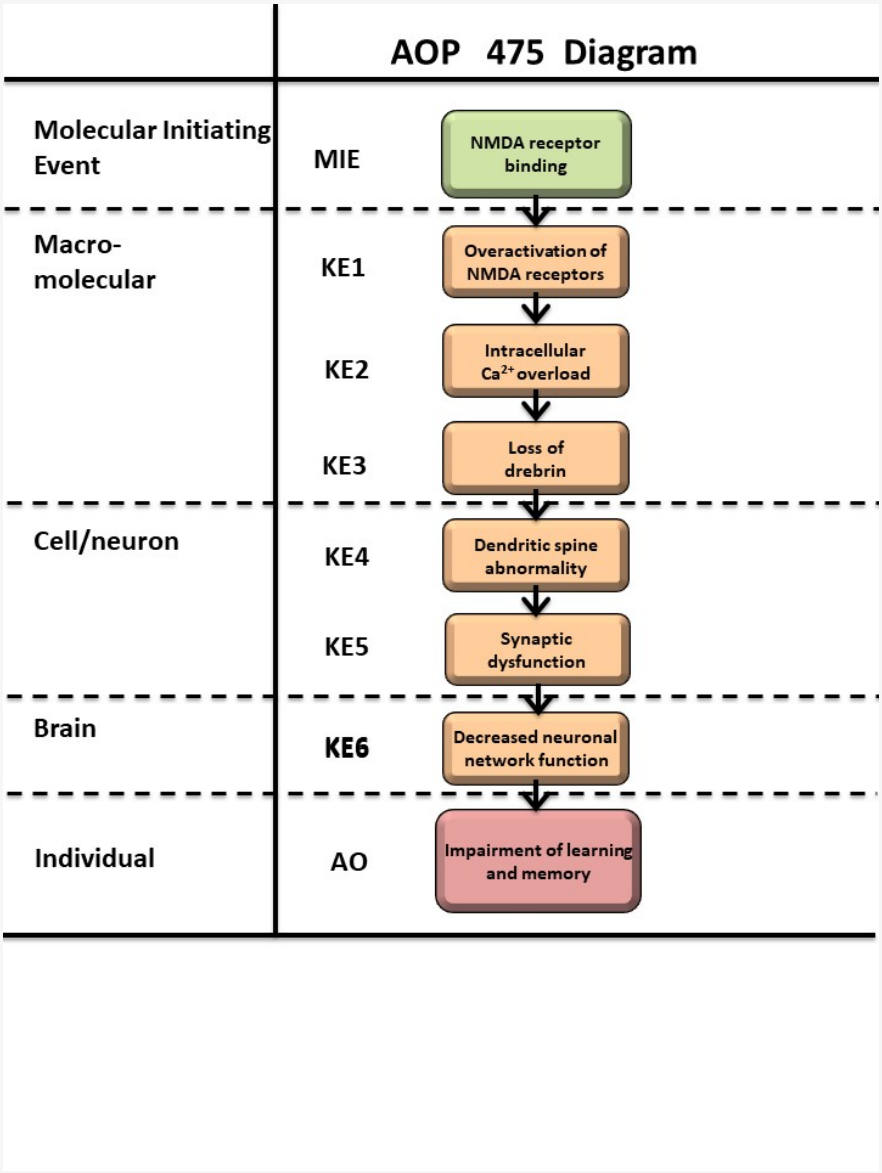
AOP ID and Title:

AOP 475: Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons
Short Title: IGR binding leads to impairment of learning and memory (via loss of drebrin)

Graphical Representation



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Abstract

Neurotoxicity risk assessment is crucial for regulatory agencies, as current methods rely on time-consuming and costly animal testing. With thousands of chemicals lacking neurotoxicity data, there is an urgent need for in vitro methods to rapidly evaluate potential risks. Chemicals that impair learning and memory are linked to neurodegenerative diseases such as Parkinson's and Alzheimer's, underscoring the importance of effective risk assessment. The proposed AOP highlights a cascade where the loss of drebrin from dendritic spines induces spine morphological

abnormalities, leading to synaptic dysfunction. Notably, synaptic dysfunction alone, even in the absence of neuronal death, can result in learning and memory impairments. This provides a novel framework for evaluating neurotoxicity and developmental neurotoxicity.

Dendritic spines are specialized structures that serve as the primary sites of excitatory synaptic transmission, primarily mediated by glutamate receptors. Spine formation and functional maturation are governed by drebrin expression. Drebrin, an actin-binding protein discovered and named by Shirao's team, has two isoforms: drebrin E (DE) and drebrin A (DA). DE, a non-neuronal protein involved in cell motility and protrusion formation, is predominantly expressed during early brain development. It is gradually replaced by DA, a neuron-specific protein that stabilizes actin filaments during synaptogenesis and synaptic maturation. The protein levels and subcellular localization of drebrin reflect its distinct roles in neuronal development and synaptic function. Loss of drebrin triggers spine abnormalities and synaptic dysfunction, ultimately impairing learning and memory as the adverse outcome (AO). This AOP builds on the molecular initiating event (MIE) of AOP 48—“Binding of agonists to ionotropic glutamate receptors”—but uniquely highlights drebrin loss as a critical KE.

Empirical evidence supports this AOP. Studies show that glutamate induces drebrin loss and dendritic spine morphological changes. Compounds that directly bind to NMDA receptors, such as glutamate and NMDA, and compounds that indirectly enhance NMDA receptor activity, have been shown to induce drebrin loss, linking such exposure to synaptic dysfunction and learning impairments. The detection of drebrin gene expression levels, subcellular localization, and protein levels provides valuable insights into brain development and higher-order functions across species. To develop effective in vitro testing methods, it is essential to have biomarkers that are simple, reproducible, and allow for quantitative data analysis. We determined that drebrin is highly suitable as a biomarker for these purposes.

The proposed AOP promotes alternative testing methods aligned with the 3Rs (Replacement, Reduction, Refinement), using cryopreserved hippocampal neurons to reduce animal use. Quantification of drebrin via immunocytochemistry and ELISA enables reproducible and scalable chemical screening. This AOP provides a foundation for in vitro prediction models, advancing chemical safety evaluation for humans and the environment. By integrating dynamic drebrin expression patterns and functional properties, this AOP framework offers a robust tool for regulatory decision-making and assessing neurotoxicity risks.

Background

Drebrin, identified by our group in 1985, has been a central focus of our research for many years. This actin-binding protein is known to exist in two isoforms: drebrin E and drebrin A. Drebrin E is expressed in both neuronal and certain non-neuronal cells, and it plays a crucial role during fetal and early postnatal stages of brain development. In neurons, drebrin E is involved in processes essential for cell motility, neurite outgrowth, and the extension of axons and dendrites, which together contribute to the establishment of neural networks. In contrast, drebrin A is a neuron-specific isoform whose expression is initiated during the synaptic formation stage. Drebrin A is indispensable for the formation and maintenance of dendritic spines, which serve as the primary sites for excitatory synaptic transmission, largely mediated by glutamate receptors. During brain development, drebrin E, which predominates in the early stages, is gradually replaced by drebrin A during synaptogenesis, reflecting their distinct roles in neuronal development, excitatory synapse formation and synaptic plasticity.

Based on the dynamic expression patterns and functional properties of drebrin, we have proposed an Adverse Outcome Pathway (AOP) framework for assessing developmental neurotoxicity and neurotoxicity. Monitoring drebrin gene expression levels, subcellular localization in neurons, and protein levels provides valuable insights into brain development and higher-order brain functions across various species.

In 2017, we summarized our research findings in a monograph titled *Drebrin: From Structure and Function to Physiological and Pathological Roles*, published as part of Springer’s *Advances in Experimental Medicine and Biology* series.

Between 2020 and 2023, we initiated the development of an AOP for neurotoxicity and developmental neurotoxicity caused by glutamate receptor-binding agonists, supported by a three-year research grant from the Japan Chemical Industry Association (JCIA) Long-range Research Initiative (LRI). This project, entitled "Proposal of a new AOP for the neurotoxicity and developmental neurotoxicity assessment of glutamate receptor binding agonists that cause learning and memory impairment," identified a novel adverse outcome (AO): learning and memory impairment. This AO is characterized by key events, including the loss of drebrin from dendritic spines, leading to thin and elongated spine morphology and subsequent synaptic dysfunction. Drebrin, as a key regulator of dendritic spine morphology, plays an essential role in the structural plasticity associated with learning and memory. Furthermore, the subcellular localization of drebrin is dynamically influenced by glutamate receptor activity.

To advance these studies, we optimized Banker’s method for low-density neuronal cultures and developed an immunocytochemical evaluation system for drebrin clusters in hippocampal neurons using a 96-well plate format and frozen embryonic hippocampal neurons from rats. In addition, we designed a high-content imaging algorithm for the quantitative analysis of neuronal parameters, including neuron count, dendrite length, and drebrin clustering, using confocal image cytometry. Notably, our brightness distribution analysis of drebrin clusters demonstrated exceptional sensitivity, allowing for precise quantification of structural changes in neurons. These methodological advancements have enabled us to establish standardized protocols (SOPs) for both neuronal culture techniques and analytical procedures.

We plan to extend this research by developing an experimental system utilizing neurons derived from human-induced pluripotent stem cells (iPSCs), thereby enhancing the relevance and applicability of our findings to human brain development and function.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	875	Binding of agonist, Ionotropic glutamate receptors	Binding of agonist, Ionotropic glutamate receptors

Sequence	Type	Event ID	Title	Short name
	KE	388	Overactivation, NMDARs	Overactivation, NMDARs
	KE	389	Increased, Intracellular Calcium overload	Increased, Intracellular Calcium overload
	KE	2078	Loss of drebrin	Loss of drebrin
	KE	2242	Abnormality, dendritic spine morphology	Dendritic spine abnormality
	KE	1944	Synaptic dysfunction	Dysfunctional synapses
	KE	386	Decrease of neuronal network function	Neuronal network function, Decreased
	AO	341	Impairment, Learning and memory	Impairment, Learning and memory

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding of agonist, Ionotropic glutamate receptors	adjacent	Overactivation, NMDARs	High	Moderate
Overactivation, NMDARs	adjacent	Increased, Intracellular Calcium overload	High	High
Increased, Intracellular Calcium overload	adjacent	Loss of drebrin	High	Moderate
Loss of drebrin	adjacent	Abnormality, dendritic spine morphology	High	High
Abnormality, dendritic spine morphology	adjacent	Synaptic dysfunction	High	High
Synaptic dysfunction	adjacent	Decrease of neuronal network function	High	Moderate
Decrease of neuronal network function	adjacent	Impairment, Learning and memory	High	Moderate
Abnormality, dendritic spine morphology	non-adjacent	Impairment, Learning and memory	High	High
Loss of drebrin	non-adjacent	Impairment, Learning and memory	High	High
Synaptic dysfunction	non-adjacent	Impairment, Learning and memory	Moderate	Moderate
Binding of agonist, Ionotropic glutamate receptors	non-adjacent	Loss of drebrin	High	High

Stressors

Name	Evidence
Sodium L-glutamate	
amyloid beta	
lotenone	
N-Methyl-D-aspartic acid	

Overall Assessment of the AOP

The overall assessment of AOP475 emphasizes its relevance in evaluating neurodevelopmental toxicity caused by chemical exposures. The pathway links disruption of dendritic spine morphology to impaired synaptic plasticity and cognitive functions, such as learning and memory. Its robust mechanistic framework integrates molecular, cellular, and behavioral endpoints. AOP475 serves as a critical tool for regulatory risk assessment, particularly in identifying early indicators of synaptic dysfunction.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Fetal	High
Perinatal	High
During brain development	High
Adult	High
Old Age	High

Life Stage	Evidence
Juvenile	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxa:
Drebrin has been primarily studied in mammals, with its expression confirmed in vertebrates such as humans, mice, rats and C-elegans.

Sex:
No sex differences in the expression or function of drebrin have been reported. Therefore, its applicability is not influenced by sex.

Life Stage:
Drebrin has two main isoforms: drebrin E, which is predominantly expressed during the fetal and juvenile stages, and drebrin A, which is specific to the mature stage. This suggests that drebrin's role varies depending on the life stage.

Essentiality of the Key Events

AOP475 describes a series of biological changes leading from the molecular initiating event (MIE), where glutamate receptor agonists bind to their receptors, to the adverse outcome (AO) of learning and memory impairment. Each Key Event (KE) plays an essential role in the progression of this pathway, and the causal relationships between these events are supported by experimental evidence and published literature. The following summarizes the Essentiality of Key Events in AOP475:

Molecular Initiating Event (MIE): Binding of agonist, Ionotropic glutamate receptors : Event ID MIE 875

This is the primary trigger for all subsequent Key Events, initiating changes in calcium homeostasis and cellular signaling. The involvement of NMDAR in neurotoxicity has been extensively documented in the literature.

KE1: Overactivation, NMDAR (KE 388)

NMDAR overactivation is a well-characterized phenomenon in excitotoxicity and has been supported by both experimental studies and reviews discussing its role in calcium dysregulation and neurodegeneration.

KE2: Increase, Intracellular Calcium overload (KE389)

Overactivation of NMDAR causes a persistent increase in intracellular calcium levels, disrupting calcium homeostasis. This dysregulation impacts cytoskeletal dynamics, including actin remodeling, and is critical for the subsequent loss of Drebrin from dendritic spines. Evidence for calcium's role in synaptic and structural stability has been widely reported in the literature.

KE3: Loss of Drebrin from Dendritic Spines (KE 2078 : a new key event)

Drebrin is essential for maintaining spine morphology and synaptic plasticity. Its loss from dendritic spines has been consistently observed in experimental models, and its significance is well-supported by publications emphasizing its role in spine stability and cognitive function.

KE4:Abnormalities Dendritic Spine Morphological (KE2242: a new key event)

Dendritic spines become thin and elongated, losing their structural stability. Morphological changes in spines impair excitatory synaptic transmission and plasticity. These abnormalities are well-documented in both experimental findings and studies on neurodegenerative diseases.

KE5: Synaptic Dysfunction (KE1944: a new key event)

Becasue denddritc spine is a structure that receive glutamate signals via glutamate receptoes. Synaptic transmission efficiency declines, reducing the ability of neurons to communicate effectively. Synaptic dysfunction, including reduced synaptic strength and plasticity, has been confirmed through electrophysiological studies and corroborated by literature focusing on the role of spines in neural networks.

KE6: Decrease of Neuronal Network Function

Network-level impairments are supported by experimental models and computational studies, as well as publications addressing the effects of synaptic disruptions on overall brain function.

Adverse Outcome (AO): Learning and Memory Impairment (AO 341)

The adverse outcome is the culmination of upstream events, supported by behavioral studies and widely recognized in reviews discussing neurotoxicity and cognitive decline.

Weight of Evidence Summary

Biological plausibility: In AOP 475, chemical stimulation of glutamate receptors, particularly NMDA receptors, results in excessive intracellular calcium influx. This calcium overload leads to a loss of drebrin from dendritic spines through several possible mechanisms,

such as translocation via acto-myosin interaction from dendritic spines to dendritic shafts, degradation by calpain, the ubiquitin-proteasome system (UPS) and caspases, calcineurin-dependent dephosphorylation leading to drebrin destabilization, and supression of drebrin synathesis through inhibition of mRNA translation. Under normal physiological conditions, drebrin binds to actin within dendritic spines, stabilizing spine morphology. Temporary drebrin reduction occurs even during physiological NMDA receptor activation and calcium influx; however, drebrin returns to dendritic spines during long-term potentiation (LTP), stabilizing newly inserted receptors and slightly enlarging spine morphology. In contrast, prolonged drebrin loss during long-term depression (LTD) changed spine morphokogy and enhances endocytosis, reducing PSD95 and glutamate receptors. Pathological conditions exacerbate drebrin loss, which is implicated in memory impairment associated with Alzheimer's disease (AD).

Empirical Support: Drebrin reduction begins during mild cognitive impairment (MCI), an early stage of AD. Experimentally induced drebrin reduction via genetic manipulation or radiation exposure results in learning and memory deficits, which can be reversed upon restoration of drebrin levels. Furthermore, animal models of Alzheimer's disease also exhibit decreased drebrin levels.

Quantitative understanding:

Existing experimental studies have quantitatively demonstrated that prolonged or excessive activation of NMDA receptors leads to measurable increases in intracellular calcium levels, which correlate with significant reductions in drebrin levels in dendritic spines. There is a quantitative relationship between the dose of glutamate applied to neuron cultures and the reduction of the number of drebrin clusters. Duration of the glutamate treatment showed shifts of the dose-response curve to the left. Quantitative data from immunocytochemical assays using cultured neurons have also shown dose-dependent decreases in drebrin cluster density following exposure to NMDA receptor agonists or neurotoxicants. Additionally, quantitative correlations between the extent of drebrin loss and the severity of cognitive impairment have been reported in animal models and clinical studies, underscoring the potential to derive quantitative thresholds or benchmarks useful in chemical risk assessment.

Quantitative Consideration

Drebrin loss has dose-response relationships to concentration of glutamate, depending on the period of the trearment.

Cognitive impairment has dode-respose to drebrin level in the brain.

Considerations for Potential Applications of the AOP (optional)

AOP475 provides a framewirk useful in risk assessments of regulatory toxicology.

KE 2078, drebrin loss, can be quantitatively measured using immunocytochemistry. Thus, targeted assays could be developed to evaluate chemicals' potential to disrupt synaptic function and cognitive performance. Quantitative measurement of the number of drebrin clusters, assessed by immunohistochemistry using neuronal cultures derived from frozen embryonic neurons, could be integrated into an IATA framework to provide a comprehensive neurotoxicity assessment, reducing reliance on animal testing.

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Appendix 1

List of MIEs in this AOP

Event: 875: Binding of agonist, Ionotropic glutamate receptors

Short Name: Binding of agonist, Ionotropic glutamate receptors

Key Event Component

Process	Object	Action
ionotropic glutamate receptor activity	ionotropic glutamate receptor complex	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	MolecularInitiatingEvent
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	MolecularInitiatingEvent

Stressors

Name
Domoic acid

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

neuron

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Drosophila melanogaster	Drosophila melanogaster	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Primates sp. BOLD:AAA0001	Primates sp. BOLD:AAA0001	High	NCBI
human	Homo sapiens	High	NCBI
mice	Mus sp.	High	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).

Key Event Description

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 depolarization of the postsynaptic neuron (Dingledine et al. 1999). Upon this depolarization the Mg2+ 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 Mg2+ 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 Mg2+ 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 depolarization 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, stabilization, degradation and post-translation modifications.

Biological compartments: The genes of the NMDAR subunits are expressed in various tissues and are not only restricted to the nervous system. The level of expression of these receptors in neuronal and non-neuronal cells depends on: transcription, chromatin remodelling, mRNA levels, translation, stabilization of the protein, receptor assembly and trafficking, energy metabolism and numerous environmental stimuli (reviewed in Traynelis et al., 2010). In hippocampus region of the brain, NR2A and NR2B are the most abundant NR2 family subunits. NR2A-containing NMDARs are mostly expressed synaptically, while NR2B-containing NMDARs are found both synaptically and extrasynaptically (Tovar and Westbrook, 1999).

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

1. They are involved in cell signalling events converting environmental stimuli to genetic changes by regulating gene transcription and epigenetic modifications in neuronal cells (Cohen and Greenberg, 2008).

2. In NMDA receptors, the ion channel is blocked by extracellular Mg2+ and Zn2+ ions, allowing the flow of Na+ and Ca2+ ions into the cell and K+ out of the cell which is voltage-dependent. Ca2+ flux through the NMDA receptor is considered to play a critical role in pre- and post-synaptic plasticity, a cellular mechanism important for learning and memory (Barria and Malinow, 2002).

3. The NMDA receptors have been shown to play an essential role in the strengthening of synapses and neuronal differentiation, through long-term potentiation (LTP), and the weakening of synapses, through long-term depression (LTD). All these processes are implicated in the memory and learning function (Barria and Malinow, 2002).

How it is Measured or Detected

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

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 Borah, 2014)

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(for Abstract and MIE)

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List of Key Events in the AOP

Event: 388: Overactivation, NMDARs

Short Name: Overactivation, NMDARs

Key Event Component

Process	Object	Action
NMDA glutamate receptor activity	NMDA selective glutamate receptor complex	increased
abnormal excitatory postsynaptic current amplitude	dendritic spine membrane	morphological change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent

AOP ID and Name	Event Type
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	MolecularInitiatingEvent
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

neuron

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Male	High

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

Key Event Description

Biological state: Please see MIE NMDARs, Binding of antagonist

Biological compartments: Please see MIE NMDARs, Binding of antagonist

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

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

Additional text, specific for this AOP:

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

Key Event Calcium influx, increased). Postsynaptic Ca2+ signals of different amplitudes and durations are able to induce either LPT or LTD.

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

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

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

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

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

How it is Measured or Detected

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

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

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

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

Microelectrode array (MEA) recordings are used to measure mainly spontaneous network activity of cultured neurons (Keefer et al., 2001, Gramowski et al., 2000 and Gopal, 2003; Johnstone et al., 2010). However, using specific agonists and antagonists of a receptor, including NMDAR, MEA technology can be used to measure evoked activity, including glutamatergic receptor function (Lantz et al., 2014). For example it has been shown that MEA-coupled neuronal cortical networks are very sensitive to pharmacological manipulation of the excitatory ionotropic glutamatergic transmission (Frega et al., 2012). MEAs can also be applied in higher throughput platforms to facilitate screening of numerous chemical compounds (McConnell et al., 2012).

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

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

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

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

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Event: 389: Increased, Intracellular Calcium overload

Short Name: Increased, Intracellular Calcium overload

Key Event Component

Process	Object	Action
calcium ion transport	calcium ion	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent
Aop:535 - Binding and activation of GPER leading to learning and memory impairments	KeyEvent
Aop:556 - Decreased Na/K ATPase activity leading to heart failure	KeyEvent
Aop:558 - Phosphodiesterase inhibition leading to heart failure	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
Human, rat, mouse	Human, rat, mouse	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Mixed	Not Specified

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

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

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

Key Event Description

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

Biological state: KE Calcium influx, Decreased

Biological compartments: KE Calcium influx, Decreased

General role in biology: KE Calcium influx, Decreased

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

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

How it is Measured or Detected

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

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

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Event: 2078: Loss of drebrin

Short Name: Loss of drebrin

Key Event Component

Process	Object	Action
postsynaptic actin cytoskeleton organization	drebrin	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

neuron

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Human, rat, mouse	Human, rat, mouse	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Unspecific	High

The results can be applied when developmental neurotoxicity and neurotoxicity in the following way.

1.Drebrin as a biomarker for Neurotoxicity: Drebrin localization in the dendritc spine of matured neuron is hightly sensitive to calcium in flux via NMDA receptors and calpain-mediated degradation. Its critical role in dendritic spine morphology and synaptic function makes it a potential biomarker for assessing neurotoxicity caused by chemical substances.

2.Evaluation of Effects on Synaptic Plasticity: The localization changes and degradation of drebrin can serve as indicators to assess the impact of chemicals on synaptic plasticity (e.g., LTP and LTD).

3.Calcium-dependent Toxicity ;In cases where chemicals induce excitotoxicity through NMDA receptor-mediated calcium influx, drebrin degradation can clarify the specific pathways and extent of neurotoxic damage.

4.Evaluation of Risk mitigation strategied; Chemicals to prevent drebrin degradation can contribute to strategies to rescue the toxicity.

5.Evolutionary Conservation of Drebrin: Drebrin's evolutionary conservation allows for the development of neurotoxicity assessment systems that can be applied across various species, including non-human models, to support broader toxicological evaluations.

Example Scenarios for Application:

- Neurotoxicity screening for newly developed chemicals.
- Risk assessment of existing chemicals that may increase the likelihood of neurodegenerative diseases.
- Providing data for safety standards in industries such as pharmaceuticals, agriculture, and industrial chemicals.

Key Event Description

Glutamate induces drebrin exodus from dendritic spines to dendritic shafts, which is reported in matured cultured neuron of rodent and human iPS-derived neurons.

NMDA-induced excitotoxicity elicits the degradation of drebrin in matured cultured neurons of rodent hippocampus and cortex.This process is triggered by calcium influx and mediated by calpains.

Drebrin is an evolutionarily conserved actin-binding protein in dendritic spines. Overexpression of drebrin A in neurons enlarges dendritic spines and decreases spine motility, whereas down-regulation of drebrin A in neurons decreases the density and width of dendritic spines and inhibits synaptic clustering of NMDARs.

Drebrin forms stable actin filaments and plays a pivotal role in dendritic spine morphogenesis (Hayashi and Shirao 1999; Takahashi et al., 2003; Takahashi et al., 2006).

During the initial stage of synaptic plasticity (either LTP or LTD), Ca^{2+} influx through the NMDA receptors arises and it brings out drebrin exodus from dendritic spines (Sekino et al., 2006).

Furthermore, prolonged NMDA-induced excitotoxicity induces calpain-mediated degradation of drebrin in vitro and in vivo.

How it is Measured or Detected

Loss of drebrin from dendritic spines can be detected by immunocytochemistry, ELISA or or Western blotting in cultured human and rodent neurons and brain tissues (Counts et al., 2006; Ishizuka et al., 2014).

The twenty-one-day primary cultured neurons were prepared using frozen stock of dissociated hippocampal neurons (Koganezawa et al, 2023; Hanamura et al 2019). In brief, cells were cultured in multi well microplates with defined medium.

For immunocytochemistry, the cultured neurons were incubated with chemicals for 1 hour. After fixation, cultured neurons were immunostained with anti-drebrin and anti-MAP2 antibodies. The cluster density of drebrin along the dendrites was automatically quantified using high content analysis instruments (Hanamura et al, 2019, Mitsuoka et al, 2019).

For enzyme-linked immunoassay, after the incubation of chemicals the extracts of neurons were quantified using ELISA kit for drebrin (ALzMEd, Inc.),

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Event: 2242: Abnormality, dendritic spine morphology

Short Name: Dendritic spine abnormality

Key Event Component

Process	Object	Action
negative regulation of synaptic plasticity	postsynaptic actin cytoskeleton	pathological

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

neuron

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Human, rat, mouse	Human, rat, mouse	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Unspecific	High

Primary cultured neuron analysis

Synaptic protein expression analysis

Spine classification:

Electrophysiological analysis

Key Event Description

Abnormalities in dendritic spines have been implicated in a wide range of psychiatric and neurological disorders, with initial discoveries stemming from Golgi staining of postmortem brains. These spine pathologies have been observed across various conditions, including schizophrenia, autism spectrum disorders (ASD), Alzheimer's disease (AD), bipolar disorder, Down syndrome, and epilepsy.Alzheimer's disease brains exhibit reduced synapse density and dendritic spine loss in the cortex and hippocampus, with greater spine loss associated with lower mental statusThis synaptic pathology is considered one of the earliest features of AD, occurring prior to neuronal loss. The shared feature of spine pathology across these disorders suggests that dendritic spines may serve as a common substrate for many brain disorders involving deficits in information processing and neuronal connectivity.

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How it is Measured or Detected

Drebrin immunocytochemistry

number of drebrin clustees

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Event: 1944: Synaptic dysfunction

Short Name: Dysfunctional synapses

Key Event Component

Process	Object	Action
decreased neurotransmitter release	CNS neuron (sensu Vertebrata)	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:429 - A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	KeyEvent
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term
principal neuronal circuit

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Human, rat, mouse	Human, rat, mouse	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Species applicability includes humans, using clinical assessments like neuroimaging, EEG, and cognitive tests; animal models (e.g., rodents) employing electrophysiological, behavioral, and biochemical assays; and cultured neuronal cells for molecular-level studies. Life-stage applicability covers developmental stages for neurodevelopmental disorders, adulthood for psychiatric and neurodegenerative diseases, and elderly populations for age-related cognitive decline. Experimental system applicability spans in vitro models for cellular mechanisms, ex vivo brain slices for electrophysiological studies, and in vivo animal models for behavioral and systemic analysis. Disease applicability involves neurological and psychiatric conditions such as Alzheimer's, Parkinson's, schizophrenia, depression, epilepsy, and autism spectrum disorders. Clearly defining these domains ensures accurate interpretation, facilitates translational research, and supports targeted therapeutic development for various neurological and psychiatric disorders.

Key Event Description

Synaptic dysfunction refers to the impairment or disruption of communication between neurons at synapses. It occurs when neurotransmitter release, receptor function, or synaptic structure is altered or damaged, leading to impaired signaling. Such dysfunction can contribute to various neurological and psychiatric conditions, including Alzheimer's disease, Parkinson's disease, depression, schizophrenia, and autism spectrum disorders.

How it is Measured or Detected

- **Electrophysiology (Patch Clamp, Field Potential Recording)**: Measures neurotransmition efficiency and receptor function by currents recordings. Evaluate synaptic plasticity, such as LTP and LTD
- **Microdialysis or HPLC (High-Performance Liquid Chromatography)**: Quantifies neurotransmitter levels directly.
- **Western Blot, ELISA**: Evaluate proteins expression.
- **Immunohistochemistry and imagings :** Evaluate expression and locarization for pre- and post synaptic proteins, mitochondrial imaging, infkammation and immune responses using microscopies.
- **Behavioral Tests (e.g., Morris Water Maze, Novel Object Recognition)**: Evaluate cognitive functions related to synaptic plasticity.
- **Morphlogical changes: Electron Microscopy (EM)**: Visualize synaptic ultrastructure. **Confocal or Two-photon Microscopy**: Observe dendritic spine morphology and density changes. **Golgi Staining**: Analyze dendritic spine density and morphology.
- **MRI, PET EEG**

Event: 386: Decrease of neuronal network function

Short Name: Neuronal network function, Decreased

Key Event Component

Process	Object	Action
synaptic signaling		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1	KeyEvent
Aop:90 - Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:405 - Organo-Phosphate Chemicals induced inhibition of AChE leading to impaired cognitive function	KeyEvent
Aop:429 - A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	KeyEvent
Aop:501 - Excessive iron accumulation leading to neurological disorders	KeyEvent

AOP ID and Name	Event Type
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	KeyEvent
Aop:522 - Estrogen antagonism leading to increased risk of autism-like behavior	KeyEvent
Aop:533 - Retinoic acid receptor antagonism during neurodevelopment leading to impaired learning and memory	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI
cat	Felis catus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

In vitro studies in brain slices applying electrophysiological techniques showed significant variability among species (immature rats, rabbits and kittens) related to synaptic latency, duration, amplitude and efficacy in spike initiation (reviewed in Erecinska et al., 2004).

Key Event Description

Biological state: There are striking differences in neuronal network formation and function among the developing and mature brain. The developing brain shows a slow maturation and a transient passage from spontaneous, long-duration action potentials to synaptically-triggered, short-duration action potentials.

Furthermore, at this precise developmental stage the neuronal network is characterised by "hyperexcitability", which is related to the increased number of local circuit recurrent excitatory synapses and the lack of γ-amino-butyric acid A (GABAA)-mediated inhibitory function that appears much later. This "hyperexcitability" disappears with maturation when pairing of the pre- and postsynaptic partners occurs and synapses are formed generating population of postsynaptic potentials and population of spikes followed by developmental GABA switch. Glutamatergic neurotransmission is dominant at early stages of development and NMDA receptor-mediated synaptic currents are far more times longer than those in maturation, allowing more calcium to enter the neurons. The processes that are involved in increased calcium influx and the subsequent intracellular events seem to play a critical role in establishment of wiring of neural circuits and strengthening of synaptic connections during development (reviewed in Erecinska et al., 2004). Neurons that do not receive glutaminergic stimulation are undergoing developmental apoptosis.

During the neonatal period, the brain is subject to profound alterations in neuronal circuitry due to high levels of synaptogenesis and gliogenesis. For example, in neuroendocrine regions such as the preoptic area-anterior hypothalamus (POA-AH), the site of gonadotropin-releasing hormone (GnRH) system is developmentally regulated by glutamatergic neurons. The changes in the expression of the N-methyl-D-aspartate (NMDA) receptor subunits NR1 and NR2B system begin early in postnatal development, before the onset of puberty, thereby playing a role in establishing the appropriate environment for the subsequent maturation of GnRH neurons (Adams et al., 1999).

Biological compartments: Neural network formation and function happen in all brain regions but it appears to onset at different time points of development (reviewed in Erecinska et al., 2004). Glutamatergic neurotransmission in hippocampus is poorly developed at birth. Initially, NMDA receptors play important role but the vast majority of these premature glutamatergic synapses are "silent" possibly due to delayed development of hippocampal AMPA receptors. In contrast, in the cerebral cortex the maturation of excitatory glutamatergic neurotransmission happens much earlier. The "silent" synapses disappear by PND 7-8 in both brain regions mentioned above.

There is strong evidence suggesting that NMDA receptor subunit composition controls synaptogenesis and synapse stabilization (Gambrill and Barria, 2011). It is established fact that during early postnatal development in the rat hippocampus, synaptogenesis occurs in parallel with a developmental switch in the subunit composition of NMDA receptors from NR2B to NR2A. It is suggested that early expression of NR2A in organotypic hippocampal slices reduces the number of synapses and the volume and dynamics of spines. In

contrast, overexpression of NR2B does not affect the normal number and growth of synapses. However, it does increase spine motility, adding and retracting spines at a higher rate. The C terminus of NR2B, and specifically its ability to bind CaMKII, is sufficient to allow proper synapse formation and maturation. Conversely, the C terminus of NR2A was sufficient to stop the development of synapse number and spine growth. These results indicate that the ratio of synaptic NR2B over NR2A controls spine motility and synaptogenesis, and suggest a structural role for the intracellular C terminus of NR2 in recruiting the signalling and scaffolding molecules necessary for proper synaptogenesis. Interestingly, it was found that genetic deletion of NR3A accelerates glutamatergic synaptic transmission, as measured by AMPAR-mediated postsynaptic currents recorded in hippocampal CA1. Consistent, the deletion of NR3A accelerates the expression of the glutamate receptor subunits NR1, NR2A, and GluR1 sugesting that glutamatergic synapse maturation is critically dependent upon activation of NMDA-type glutamate receptors (Henson et al., 2012).

General role in biology: The development of neuronal networks can be distinguished into two phases: an early ‘establishment’ phase of neuronal connections, where activity-dependent and independent mechanisms could operate, and a later ‘maintenance’ phase, which appears to be controlled by neuronal activity (Yuste and Sur, 1999). These neuronal networks facilitate information flow that is necessary to produce complex behaviors, including learning and memory (Mayford et al., 2012).

How it is Measured or Detected

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

In vivo: The recording of brain activity by using electroencephalography (EEG), electrocorticography (ECoG) and local field potentials (LFP) assists towards the collection of signals generated by multiple neuronal cell networks. Advances in computer technology have allowed quantification of the EEG and expansion of quantitative EEG (qEEG) analysis providing a sensitive tool for time-course studies of different compounds acting on neuronal networks' function (Binienda et al., 2011). The number of excitatory or inhibitory synapses can be functionally studied at an electrophysiological level by examining the contribution of glutamatergic and GABAergic synaptic inputs. The number of them can be determined by variably clamping the membrane potential and recording excitatory and inhibitory postsynaptic currents (EPSCs or IPSCs) (Liu, 2004).

In vitro: Microelectrode array (MEA) recordings are also used to measure electrical activity in cultured neurons (Keefer et al., 2001, Gramowski et al., 2000; Gopal, 2003; Johnstone et al., 2010). MEAs can be applied in high throughput platforms to facilitate screening of numerous chemical compounds (McConnell et al., 2012). Using selective agonists and antagonists of different classes of receptors their response can be evaluated in a quantitative manner (Novellino et al., 2011; Hogberg et al., 2011).

Patch clamping technique can also be used to measure neuronal network activity.In some cases, if required, planar patch clamping technique can also be used to measure neuronal networks activity (e.g., Bosca et al., 2014).

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List of Adverse Outcomes in this AOP

Event: 341: Impairment, Learning and memory

Short Name: Impairment, Learning and memory

Key Event Component

Process	Object	Action
learning		decreased
memory		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	AdverseOutcome
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	AdverseOutcome
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	AdverseOutcome
Aop:77 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1	KeyEvent
Aop:87 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure	KeyEvent
Aop:88 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure via abnormal role change within caste	KeyEvent
Aop:89 - Nicotinic acetylcholine receptor activation followed by desensitization contributes to abnormal foraging and directly leads to colony loss/failure	KeyEvent
Aop:90 - Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2	KeyEvent
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	AdverseOutcome
Aop:99 - Histamine (H2) receptor antagonism leading to reduced survival	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	AdverseOutcome
Aop:475 - Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	AdverseOutcome
Aop:483 - Deposition of Energy Leading to Learning and Memory Impairment	AdverseOutcome
Aop:490 - Co-activation of IP3R and RyR leads to reduced IQ through non-cholinergic mechanisms	AdverseOutcome
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	AdverseOutcome
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	AdverseOutcome
Aop:520 - Retinoic acid receptor agonism during neurodevelopment leading to impaired learning and memory	AdverseOutcome
Aop:525 - Reduced oligodendrocyte differentiation during neurodevelopment leading to impaired learning and memory	AdverseOutcome
Aop:533 - Retinoic acid receptor antagonism during neurodevelopment leading to impaired learning and memory	AdverseOutcome
Aop:535 - Binding and activation of GPER leading to learning and memory impairments	AdverseOutcome

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

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

Life Stage Applicability

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

Sex Applicability

Sex	Evidence
Mixed	High

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

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

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

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

Key Event Description

(Adapted from KE: 341 - in blue)

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

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

Learning and memory depend upon the coordinated action of different brain regions and neurotransmitter systems constituting functionally integrated neural networks (D'Hooge and DeDeyn, 2001). Among the many brain areas engaged in the acquisition of, or retrieval of, a learned event, the hippocampal-based memory systems have received the most study. For example, the hippocampus has been shown to be critical for spatial-temporal memory, visio-spatial memory, verbal and narrative memory, and episodic and autobiographical memory (Burgess et al., 2000; Vorhees and Williams, 2014). However, there is substantial evidence that fundamental learning and memory functions are not mediated by the hippocampus alone but require a network that includes, in addition to the hippocampus, anterior thalamic nuclei, mammillary bodies cortex, cerebellum and basal ganglia (Aggleton and Brown, 1999; Doya, 2000; Mitchell et al., 2002, Toscano and Guilarte, 2005; Gilbert et al., 2006, 2016). Thus, damage to variety of

brain structures can potentially lead to impairment of learning and memory. The main learning areas and pathways are similar in rodents and primates, including man (Eichenbaum, 2000; Stanton and Spear, 1990). While the prefrontal cortex and frontostriatal neural circuits have been identified as the primary sites of higher-order cognition in vertebrates, invertebrates utilize paired mushroom bodies, shown to contain ~300,000 neurons in honey bees (Menzel, 2012; Puig et al., 2014).

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

How it is Measured or Detected

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

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

Novel Object recognition. This is a simpler task that can be used to probe recognition memory. Two objects are presented to animal in an open field on trial 1, and these are explored. On trial 2, one object is replaced with a novel object and time spent interacting with the novel object is taken evidence of memory retention – I have seen one of these objects before, but not this one (Cohen and Stackman, 2015).

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

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

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

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

Modifications have been made and norms developed for incorporating of tests of learning and memory in children. Examples of some of these tests include:

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

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

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

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

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

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

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

In Honey Bees: For over 50 years an assay for evaluating olfactory conditioning of the proboscis extension reflex (PER) has been used as a reliable method for evaluating appetitive learning and memory in honey bees (Guirfa and Sandoz, 2012; LaLone et al., 2017). These experiments pair a conditioned stimulus (e.g., an odor) with an unconditioned stimulus (e.g., sucrose) provided immediately afterward, which elicits the proboscis extension (Menzel, 2012). After conditioning, the odor alone will lead to the conditioned PER. This methodology has aided in the elucidation of five types of olfactory memory phases in honey bee, which include early short-term memory, late short-term memory, mid-term memory, early long-term memory, and late long-term memory (Guirfa and Sandoz, 2012). These phases are dependent on the type of conditioned stimulus, the intensity of the unconditioned stimulus, the number of conditioning trials, and the time between trials. Where formation of short-term memory occurs minutes after conditioning and decays within minutes, memory consolidation or stabilization of a memory trace after initial acquisition leads to

mid-term memory, which lasts 1 d and is characterized by activity of the cAMP-dependent PKA (Guirfa and Sandoz, 2012). Multiple conditioning trials increase the duration of the memory after learning and coincide with increased Ca2+-calmodulin-dependent PKC activity (Guirfa and Sandoz, 2012). Early long-term memory, where a conditioned response can be evoked days to weeks after conditioning requires translation of existing mRNA, whereas late long-term memory requires de novo gene transcription and can last for weeks (Guirfa andSandoz, 2012)."

Regulatory Significance of the AO

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

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

al., 2012).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 919: Binding of agonist, Ionotropic glutamate receptors leads to Overactivation, NMDARs

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	adjacent	High	
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Human, rat, mouse	Human, rat, mouse	High	NCBI
human and other cells in culture	human and other cells in culture	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Unspecific	High

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 characterized in the existing literature.

Key Event Relationship Description

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 depolarization of the postsynaptic neuron (as descibed in MIE). Upon this depolarization the Mg2+ 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.

Evidence Supporting this KER

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 depolarization of postsynaptic neurons that relieves the block of the channel pore by Mg2+, 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 desensitization. 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 Ca2+, 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 Evidence

Include consideration of temporal concordance here

There is well established understanding of NMDAR activation by endogenous agonist glutamate that happens in the absence of the Mg2+ 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 et al., 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 “memorizing” 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 localized 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 depolarization, which would bring the neuron closer to its spike firing threshold. By having this dual localization, 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 Mg2++ block because of domoic acid–induced depolarization. The final event is an increase of NMDA-mediated Ca2++ 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 and 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.

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

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	adjacent	Moderate	
Acetylcholinesterase Inhibition Leading to Neurodegeneration	adjacent	High	Moderate
Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	adjacent	Not Specified	Not Specified
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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

Key Event Relationship Description

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

Evidence Supporting this KER

Biological Plausibility

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

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

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

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

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

the appropriate chemical cues for desensitization are blocked. The excess intracellular Ca2+ causes disruption of cellular function, cell swelling and ultimately cell death (Lefebvre and Robertson,2010).

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

Empirical Evidence

Include consideration of temporal concordance here

Domoic acid (DomA)

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

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

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

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

Stressor	Experimental Model	Tested concentrations	Exposure route	Exposure duration	Overactivation of NMDAR (KE up) (measurements, quantitative if available)	Increased intracelllular Ca 2+ levels (KE down) (measurements, quantitative if available)	References	Temporal Relationship	Dose-response relationship	Incidence	Comments
DomA	Mouse cerebellar granule neurons (CGNs) from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 μM		Time course (15 to 120 min)		5 and 8 fold increase of [Ca2+]i compared to controls.	Giordano et al., 2006				The cells were exposed simultaneously to DA and the NMDA receptor antagonist MK-801 and the Ca2+ levels were found to be close to control levels, indicating that the Ca2+ elevation evoked by DA involves activation of NMDA receptors.

DomA	CGNs from Gclm (+/+) and Gclm (−/−) mice	0.01 to 10 µM		Time course (0 to 25 min)		0.1µM domoic acid caused a small and delayed increase (4 fold) in [Ca2+]i, with a full recovery by 20 min.In contrast, the higher concentration of domoic acid (10µM) caused a rapid and robust increase (8 fold) in [Ca2+]i, which was still elevated after 25 min. 0.1µM DA increases [Ca2+]M by about 3 fold, with a delay of about 15 min. In contrast, no changes in [Ca2+]M were observed following 10µM of DA.	Giordano et al., 2007				At the low concentration (0.1µM), the recording of intracellular Ca2+ was antagonized only by NBQX (AMPA receptor antagonist), but not by MK-801 (NMDA receptor antagonist). On the other hand, the higher DA concentration (10µM) caused a rapid and robust increase in intracellular Ca2+ . This effect was antagonized by both NBQX and MK-801, suggesting the importance of NMDA receptors in Ca2+ elevation evoked by DA but only at high doses
DomA	10-13 DIV CGNs obtained from 8-day-old Sprague–Dawley rats	0.1 to 30 µM		Time course (0 to 45 min)		EC50 for DA-induced increase in intracellular Ca2+ was 0.61 µM	Berman et al., 2002				The NMDA receptor antagonist MK-801 significantly reduced both peak and final plateau of intracellular Ca2+ by 30 and 70%, respectively
DomA	Adult male Sprague Dawley rats	10 µM	Brain microinjection			Increased phosphorylated CaMKII and phosphorylated CREB levels	Qiu and Currás-Collazo, 2006				
Glutamate, NMDA	Mouse cortical astrocytes	Glutamate: 100 µM NMDA: 20 µM		Brief (1s application)		Increased intracellular Ca2+ measured through Fluo-3 Flouresence	Palygin et al., 2011				Provides time-series data of intracellular calcium measured through fluoresence given an application of Glu, NMDA, or Glu + D-AP5 in mouse cortical astrocytes. Cells were additionally exposed to D-AP5, an NMDA antagonist, and showed reduced fluorscence changes. (added by DS for AOP 281)
Glutamate	Cultured rat hippocampal neurons	500 µM		Time course (0 to 45 minutes)		Increased intracellular Ca2+ measured through Fura-2 Flourescence	Michaels and Rothman, 1990				Provides time-series data of intracellular calcium measured through fluoresence, as well as directly providing calculated intracellular calcium concentrations in response to high concentrations of applied Glu, both alone and with antagonists. (added by DS for AOP 281)

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

Glufosinate (GLF)

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

Uncertainties and Inconsistencies

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

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Relationship: 3091: Increased, Intracellular Calcium overload leads to Loss of drebrin

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Fetal	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Key Event Relationship Description

NMDA receptor overactivation elevates intracellular calcium leads to loss of drebrin from dendritic spines of cultured coltcal neurons and hippocampal neurons.

Relationship: 3298: Loss of drebrin leads to Dendritic spine abnormality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human, mouse, rat	human, mouse, rat	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	Not Specified

Key Event Relationship Description

Drebrin loss alters spine shape by dismantling key protein–actin and receptor trafficking interactions.

Relationship: 3301: Dendritic spine abnormality leads to Dysfunctional synapses

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent	High	High

Relationship: 2504: Dysfunctional synapses leads to Neuronal network function, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
A cholesterol/glucose dysmetabolism initiated Tau-driven AOP toward memory loss (AO) in sporadic Alzheimer's Disease with plausible MIE's plug-ins for environmental neurotoxicants	adjacent	High	
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent	High	Moderate

Relationship: 359: Neuronal network function, Decreased leads to Impairment, Learning and memory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2	adjacent		
Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1	adjacent		
Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	adjacent	High	Low
Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	adjacent	High	
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	adjacent	Low	
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

Synaptic transmission and plasticity are achieved via mechanisms common across taxonomies. LTP has been recorded in aplysia, lizards, turtles, birds, mice, guinea pigs, rabbits and rats. Deficiencies in hippocampally based learning and memory following developmental hypothyroidism have been documented mainly in rodents and humans.

Key Event Relationship Description

Learning and memory is one of the outcomes of the functional expression of neurons and neural networks from mammalian to invertebrates. Damage or destruction of neurons by chemical compounds during development when they are in the process of synapses formation, integration and formation of neural networks, will derange the organization and function of these networks, thereby setting the stage for subsequent impairment of learning and memory. Exposure to the potential developmental toxicants during neuronal differentiation and synaptogenesis will increase risk of functional neuronal network damage leading to learning and memory impairment.

Impairments in learning and memory are measured using behavioral techniques. It is well accepted that these alterations in behavior are the result of structural or functional changes in neurocircuitry. Functional impairments are often measured using field potentials of critical synaptic circuits in hippocampus and cortex. A number of studies have been performed in rodent models that reveal deficits in both excitatory and inhibitory synaptic transmission in the hippocampus as a result of developmental thyroid insufficiency (Wang et al., 2012; Oerbeck et al., 2003; Wheeler et al., 2011; Wheeler et al., 2015; Willoughby et al., 2014; Davenport and Dorcey, 1972; Tamasy et al., 1986; Akaike, 1991; Axelstad et al., 2008; Gilbert and Sui, 2006; Gilbert et al., 2016; Gilbert, 2011; Gilbert et al., 2016). A well-established functional readout of memory at the synaptic level is known as long-term potentiation (LTP) (i.e., a persistent strengthening of synapses based on recent patterns of activity). Deficiencies in LTP are generally regarded as potential substrates of learning and memory impairments. In rodent models where synaptic function is impaired by TH deficiencies, deficits in hippocampus-mediated memory are also prevalent (Gilbert and Sui, 2006; Gilbert et al., 2016; Gilbert, 2011; Gilbert et al., 2016).

Evidence Supporting this KER

A number of studies have consistently reported alterations in synaptic transmission resulting from developmental TH disruption, and leading to decreased cognition.

Biological Plausibility

Long-term potentiation (LTP) is a long-lasting increase in synaptic efficacy and its discovery suggested that changes in synaptic strength could provide the substrate for learning and memory (reviewed in Lynch, 2004). 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 excitatory postsynaptic currents (EPSCs) to be developed quickly and to be persistent for few weeks at least 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, 2004).

A review on Morris water maze (MWM) as a tool to investigate spatial learning and memory in laboratory rats also pointed out that the disconnection between neuronal networks rather than the brain damage of certain regions is responsible for the impairment of MWM performance. Functional integrated neural networks that involve the coordination action of different brain regions are consequently important for spatial learning and MWM performance (D'Hooge and De Deyn, 2001).

Moreover, it is well accepted that alterations in synaptic transmission and plasticity contribute to deficits in cognitive function. There are a number of studies that have linked exposure to TPO inhibitors (e.g., PTU, MMI), as well as iodine deficient diets, to changes in serum TH levels, which result in alterations in both synaptic function and cognitive behaviors (Akaike et al., 1991; Vara et al., 2002; Gilbert and Sui, 2006; Axelstad et al., 2008; Taylor et al., 2008; Gilbert, 2011; Gilbert et al., 2016), described in the indirect KER "Decrease of TH synthesis leads to learning and memory deficits".

Empirical Evidence

Developmental hypothyroidism reduces the functional integrity in brain regions critical for learning and memory. Neurophysiological indices of synaptic transmission of excitatory and inhibitory circuitry are impaired in the hippocampus of hypothyroid animals. Both hippocampal regions (area CA1 and dentate gyrus) exhibit alterations in excitatory and inhibitory synaptic transmission following reductions in serum TH in the pre and early postnatal period (Vara et al., 2002; Sui and Gilbert, 2003; Sui et al., 2005; Gilbert and Sui, 2006; Taylor et al., 2008; Gilbert, 2011; Gilbert et al., 2016). These alterations persist into adulthood despite a recovery to euthyroid conditions in blood. The latter observation indicates that these alterations represent permanent changes in brain function caused by transient hormones insufficiencies induced during critical window of development.

Because the adult hippocampus is involved in learning and memory, it is a brain region of remarkable plasticity. Use-dependent synaptic plasticity is critical during brain development for synaptogenesis and fine tuning of synaptic connectivity. In the adult brain, similar plasticity mechanisms underlie use-dependency that underlies learning and memory, as exhibited in LTP model of synaptic memory. Hypothyroidism during development reduces the capacity for synaptic plasticity in juvenile and adult offspring (Vara et al., 2002; Sui and Gilbert, 2003; Dong et al., 2005; Sui et al., 2005; Gilbert and Sui, 2006; Taylor et al., 2008; Gilbert, 2011; Gilbert et al., 2016). Decrease of neuronal network function and plasticity are observed coincident with deficits in learning tasks that require the hippocampus.

- Wang et al., 2012: This study showed that maternal subclinical hypothyroidism impairs spatial learning in the offspring, as well as the efficacy and optimal time of T4 treatment in pregnancy. Female adult Wistar rats were randomly divided into six groups: control, hypothyroid (H), subclinical hypothyroid (SCH) and SCH treated with T4, starting from GD10, GD13 and GD17, respectively, to restore normal TH levels. Results indicate that progenies of SCH and H groups demonstrated significantly longer mean latency in the water maze test (on the 2^{nd} training day, latency was ~83% higher in H group, and ~50% higher in SCH), and a lower amplification percentage of the amplitude (~15% lower in H group, and 12% lower in SCH), and slope of the field excitatory postsynaptic potential (fEPSP) recording (~20% lower in H group, and 17% lower in SCH), compared to control group. T4 treatment at GD10 and GD13 significantly shortened mean latency and increased the amplification percentage of the amplitude and slope of the fEPSPs of the progeny of rats with subclinical hypothyroidism. However, T4 treatment at GD17 showed only minimal effects on spatial learning in the offspring. Altogether these data indicate direct correlation between decrease of neural network function and learning and memory deficits.

- Liu et al., 2010 This study assessed the effects of hypothyroidism in 60 female rats who were divided into three groups: (i) maternal subclinical hypothyroidism (total thyroidectomy with T4 infusion), (ii) maternal hypothyroidism (total thyroidectomy without T4 infusion), and (iii) control (sham operated). The Morris water maze tests revealed that pups from the subclinical hypothyroidism group showed long-term memory deficits, and a trend toward short-term memory deficits.

- Gilbert and Sui, 2006 Administration of 3 or 10 ppm PTU to pregnant and lactating dams via the drinking water from GD6 until PND30 caused a 47% and 65% reduction in serum T4, in the dams of the low and high-dose groups, respectively. Baseline synaptic transmission was impaired in PTU-exposed animals: mean EPSP slope (by ~60% with 10 ppm PTU) and population spike amplitudes (by ~70% with 10 ppm PTU) in the dentate gyrus were reduced in a dose-dependent manner in adult offspring of PTU-treated dams. High-

dose animals (10 ppm) demonstrated very little evidence of learning despite 16 consecutive days of training (~5-fold higher mean latency to find the hidden platform, used as an index of learning).

- Gilbert et al., 2016 Exposure to PTU during development produced dose-dependent reductions in mRNA expression of nerve growth factor (Ngf) in whole hippocampus of neonates. These changes in basal expression persisted to adulthood despite the return to euthyroid conditions in blood. Developmental PTU treatment dramatically reduced the activity-dependent expression of neurotrophins and related genes in neonate hippocampus and was accompanied by deficits in hippocampal-based learning (e.g., mean latency to find a hidden platform, at 2nd trial resulted ~60% higher in rats treated with 10 ppm PTU).

- Gilbert, 2011 Trace fear conditioning deficits to context and to cue reported in animals treated with PTU and who also displayed synaptic transmission and LTP deficits in hippocampus. Baseline synaptic transmission was impaired in PTU-exposed animals (by ~50% in animal treated with 3 ppm PTU). EPSP slope amplitudes in the dentate gyrus were reduced in a dose-dependent manner in adult offspring of PTU-treated dams.

BPA, an environmental toxicant known to inhibit NIS-mediated iodide uptake (Wu Y et al., 2016) has been found to cause learning and memory deficits in rodents as described below:

- **Jang et al., 2012** In this study, pregnant female C57BL/6 mice (F0) were exposed to BPA (0.1-10 mg/kg) from gestation day 6 to 17, and female offspring (F2) from F1 generation mice were analysed. Exposure of F0 mice to BPA (10 mg/kg) decreased hippocampal neurogenesis (~ 30% decrease of hippocampal BrdU^{+} cells vs control) in F2 female mice. High-dose BPA (10 mg/kg) caused neurocognitive deficit (i.e., reduced memory retention) as shown by passive avoidance testing (~ 33% decrease vs control) in F2 mice. Furthermore, 10 mg/kg BPA decreased the hippocampal levels of BDNF (~ 35% lower vs control) in F2 mice. These results suggest that BPA exposure (NIS inhibitor) in pregnant mothers could decrease hippocampal neurogenesis (decreased number of neurons) and cognitive function in future generations.

In humans, the data linking these two specific KE are much more limited, but certainly clear reductions in IQ, with specific impairments in hippocampus-mediated functions have been observed.

- Wheeler et al., 2015 This study assessed hippocampal functioning in adolescents with congenital hypothyroidism (CH), using functional magnetic resonance imaging (fMRI). 14 adolescents with CH and 14 typically developing controls (TDC) were studied. Hippocampal activation was greater for pairs than items in both groups, but this difference was only significant in TDC. When the groups were directly compared, the right anterior hippocampus was the primary region in which the TDC and CH groups differed for this pair memory effect. Results signify that adolescents with CH show abnormal hippocampal functioning during verbal memory processing, in order to compensate for the effects induced by TH deficit in the brain.

- Wheeler et al., 2012 In this study hippocampal neuronal network function was measured based on synaptic performance using fMRI and was altered while subjects engaged in a memory task. Data showed paired word recognition deficits in adolescents with congenital hypothyroidism (N = 14; age range, 11.5-14.7 years) compared with controls (N = 15; age range, 11.2-15.5 years), with no impairment on simple word lists. Analysis of functional magnetic resonance imaging showed that adolescents with congenital hypothyroidism had both increased magnitude of hippocampal activation relative to controls and bilateral hippocampal activation when only the left was observed in controls. Furthermore, the increased activation in the congenital hypothyroidism group was correlated with the severity of the hypothyroidism experienced early in life.

- Willoughby et al., 2013 Analogously, in this study, fMRI revealed increased hippocampus activation with word pair recognition task in CH and children born to women with hypothyroxinemia during midgestation. These differences in functional activation were not seen with single word recognition, but were revealed when retention of word pair associations was probed. The latter is a task requiring engagement of the hippocampus.

A series of important findings suggest that the biochemical changes that happen after induction of LTP also occur during memory acquisition, showing temporality between the two KEs (reviewed in Lynch, 2004).

- Morris et al., 1986 This study found that blocking the NMDA receptor of the neuronal network with AP5 inhibits spatial learning in rats. Most importantly, in the same study they measured brain electrical activity and recorded that this agent also inhibits LTP, however, they have not proven that spatial learning and LTP inhibition are causally related.

Since then a number of NMDA receptor antagonists have been studied towards their ability to induce impairment of learning and memory. It is worth mentioning that similar findings have been found in human subjects:

- Grunwald et al., 1999 By combining behavioural and electrophysiological data from patients with temporal lobe epilepsy exposed to ketamine, involvement of NMDA receptors in human memory processes was demonstrated.

The last KE preceding the AO (learning and memory deficits), i.e. "Decreased Neural Network Function", is also common to the AOP 13, entitled "Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities" (https://aopwiki.org/aops/13). In this AOP 13, data on lead (Pb) exposure as reference chemical are reported. While these studies do not refer to TH disruption, they provide empirical support for the same KER described in the present AOP.

Pb2+: Exposure to low levels of Pb2+, during early development, has been implicated in long-lasting behavioural abnormalities and cognitive deficits in children (Needleman et al., 1975; Needleman and Gatsonis, 1990; Bellinger et al., 1991; 1992; Baghurst et al., 1992; Leviton et al., 1993; Needleman et al., 1996; Finkelstein et al., 1998; Lanphear et al., 2000; 2005; Canfield et al., 2003; Bellinger 2004; Lanphear et al., 2005; Surkan et al., 2007; Jusko et al., 2008; Neal and Guilarte, 2010) and experimental animals (Brockel and Cory-Slechta, 1998; Murphy and Regan, 1999; Moreira et al., 2001). Multiple lines of evidence suggest that Pb2+ can impair hippocampus-mediated learning in animal models (reviewed in Toscano and Guilarte, 2005).

- Jett et al., 1997 Female rats exposed to Pb^{2+} through gestation and lactation have shown more severe impairment of memory than male rats with similar Pb^{2+} exposures.

- De Souza Lisboa et al., 2005 This study reported that exposure to Pb^{2+} during both pregnancy and lactation caused depressive-like behaviour (detected in the forced swimming test) in female but not male rats.

- Anderson et al., 2012 This study investigated the neurobehavioral outcomes in Pb^{2+}-exposed rats (250, 750 and 1500 ppm Pb^{2+} acetate in food) during gestation and through weaning and demonstrated that these outcomes are very much influenced by sex and rearing environment. In females, Pb^{2+} exposure lessened some of the benefits of enriched environment on learning, whereas, in males, enrichment does help to overcome detrimental effects of Pb^{2+} on learning. Regarding reference memory, environmental enrichment has not been beneficial in females when exposure to Pb^{2+} occurs, in contrast to males.

- Jaako-Movits et al., 2005 Wistar rat pups were exposed to 0.2% Pb^{2+} via their dams' drinking water from PND 1 to PND 21 and directly via drinking water from weaning until PND 30. At PND 60 and 80, the neurobehavioural assessment has revealed that developmental Pb^{2+} exposure induces persistent increase in the level of anxiety and inhibition of contextual fear conditioning. The same behavioural syndrome in rats has been described in Salinas and Huff, 2002.

- Finkelstein et al., 1998 These observations are in agreement with observations on humans, as children exposed to low levels of Pb^{2+} displayed attention deficit, increased emotional reactivity and impaired memory and learning.

- Kumar and Desiraju, 1992 In Wistar rats fed with lead acetate (400 µg/g body weight/day) from PND 2 until PND 60, EEG findings showed statistically significant reduction in the delta, theta, alpha and beta band EEG spectral power in motor cortex and hippocampus, but not in delta and beta bands power of motor cortex in wakeful state. After 40 days of recovery, animals were assessed for their neurobehaviour, and revealed that Pb^{2+} treated animals showed more time and sessions in attaining criterion of learning than controls.

Further data obtained using animal behavioral techniques demonstrate that NMDA mediated synaptic transmission is decreased by Pb^{2+} exposure (Cory-Slechta, 1995; Cohn and Cory-Slechta, 1993 and 1994).

- Xiao et al., 2014 Rat pups from parents exposed to 2 mM $PbCl_2$ three weeks before mating until their weaning (pre-weaning Pb^{2+}) and weaned pups exposed to 2 mM $PbCl_2$ for nine weeks (post-weaning Pb^{2+}) were assessed for their spatial learning and memory by MWM on PND 85-90. The study revealed that both rat pups in pre-weaning Pb^{2+} and post-weaning Pb^{2+} groups performed significantly worse than those in the control group. The number of synapses in pre-weaning Pb^{2+} group increased significantly, but it was still less than that of control group. The number of synapses in post-weaning Pb^{2+} group was also less than that of control group, although the number of synapses had no differences between post-weaning Pb^{2+} and control groups before MWM. In both pre-weaning Pb^{2+} and post-weaning Pb^{2+} groups, synaptic structural parameters such as thickness of postsynaptic density (PSD), length of synaptic active zone and synaptic curvature increased, whereas width of synaptic cleft decreased compared to controls.

The last KE preceding the AO (learning and memory deficits), i.e. "Decreased Neural Network Function", is also common to the AOP 17, entitled " Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins during brain development leads to impairment of learning and memory" (https://aopwiki.org/aops/17). In this AOP 17, data on mercury exposure as reference chemical are reported. While these studies do not refer to TH disruption, they provide empirical support for the same KER described in the present AOP.

Sokolowski et al. 2013. Rats at postnatal day 7 received a single injection of methylmercury (0.6 microgr/g, that caused caspase activation in the hilus of granule cell layer in hippocampus. At PD 21, a decrease in cell number or 22% in hilus and of 27% in granule cell layer, as well as a decreased proliferation of neural precursor cells of 25% were observed. This was associated with a decrease of spatial memory as assessed by Morris water maze.

Eddins et al., 2008. Mice exposed during postnatal week 1-3 to 2-5 mg/kg mercury chloride in 0.01 ml/g of NaCl injectd s.c. The behavioral tests at 3 months of age revealed learning deficits (radial maze), which was associated with increased levels of monoamines in frontal cortex.

Zanoli et al., 1994. Single injection of methylmercury (8 mg/kg by gavage) at gestational day 15. Offsprings analyzed at 14, 21, and 60 days of age exhibited a decrease in the number of muscarinic receptors at 14 and 21 days and a decrease in avoidance latency at 60 days, indicating learning and memory deficits.

Zanoli et al., 2001. Single injection of methylmercury (8 mg/kg) at gestational day 8. Brain was removed at PD 21 and 60. An increase in tryptophan level in hippocampus was detected at both days. At PD 21, a decrease in anthranilic acid and an increase in quinolinic acid was found. No change in glutamic acid nor in aspartic acid were detected.

Montgomery et al., 2008. C57/B6 mice exposed during pregnancy (GD 8-18) with food containing methylmercury (0.01 mg/kg body wheight). Tested when adult, they showed deficits in motor function, coordination, overall activity and impairment in reference memory.

Glover et al., 2009. Balb mice exposed to methylmercury in diet (low dose: 1.5 mg/kg; high dose: 4.5 mg/kg) during 11 weeks (6 weeks prior mating, 3 weeks during gestation and 2 weeks post-partum). Offsprings tested at PD 15 showed an accumulation of Hg in brain (0.08 mg/kg for low dose and 0.25 mg/kg for the high dose). At hte cellular level, there was alterations in gene expression for cytoskeleton, cell processes, cell adhesion, cell differentiation, development), which could be all involved in cellular network formation. This was associated with behavioral impairment, i.e. a decrease in exploratory activity measured in open field.

Onishchenko et al., 2007. Pregnant mice received 0.5 mg methylmercury/kg/day in drinking water from gestational dy 7 until day 7 after delivery. Offspring behavior was monitored at 5-15 and 26-36 weeks of age. Mercury-induced alterations in reference memory were detected.

Cagiano et al., 1990. Pregnant rat received at GD 15 8mg/kg of methylmercury by gavage. Offsprings were tested at day 16, 21 and 60. A reduced functional activity of glutamatergic system associated with disturbances in learning and memory were observed.

Rice, 1992. Female monkeys exposed to 10, 25 and 50 microg/kg/day to methylmercury. Male unexposed. Infants separated from mother at birth and exposed to similar doses did not show gross intellectual impairment, but interferences with temporal discrimination.

Sahin et al., 2016. Exposure of rat pups for 5 weeks or 5 months with mercury chloride (4.6 microg/kg as first injection, followed each day by 0.07 microg/kg/day). Learning and memory impairment measured by passive avoidance and Morris-water-maze was found in 5-weeks group, but not in the 5-month group. This was accompanied by hearing loss.

In humans:

Orenstein et al., 2014. Maternal peripartum hair mercury level was measured to assess prenatal mercury exposure. The concentrations of mercury was found in the range of 0.3-5.1 microg/g, similar to fish eating population in US. However, statistical analyses revealed that each microg/g increase in hair Hg was associated with a decrement in visula memory, learning and verbal memory.

Yorifuji et al., 2011. A survey of the Minamata exposed population made in 1971 to assess pre- and post-natal exposure revealed a methylmercury-induced impairment of intelligence as well as behavioral dysfunction.

Uncertainties and 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 what modifications of 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.

The direct relationship of alterations in neural network function and specific cognitive deficits is difficult to ascertain given the many forms that learning and memory can take and the complexity of synaptic interactions in even the simplest brain circuit. Linking of neurophysiological assessments to learning and memory processes have, by necessity, been made across simple monosynaptic connections and largely focused on the hippocampus. Alterations in synaptic function have been found in the absence of behavioral impairments. This may result from measuring only one component in the complex brain circuitry that underlies 'cognition', behavioral tests that are not sufficiently sensitive for the detection of subtle cognitive impairments, and behavioral plasticity whereby tasks are solved by the animal via different strategies developed as a consequence of developmental insult.

Finally, in order to provide empirical support for this KER, data on the effects of lead (Pb) exposure are reported. Several epidemiological studies where Pb2+ exposure levels have been studied in relation to neurobehavioural alterations in children have been reviewed in Koller et al. 2004. This review has concluded that in some occasions there is negative correlation between Pb2+ dose and cognitive deficits of the subjects due to high influence of social and parenting factors in cognitive ability like learning and memory (Koller et al. 2004), meaning that not always Pb2+ exposure is positively associated with learning and memory impairment in children.

Mercury

Olczak et al., 2001. Postnatal exposure of rats to Thimerosal (4 injections with 12, 240, 1440 and 3000 microgHg/kg per injection). Effects were measured in adult, which exhibited alterations in dopaminergic system with decline in the density of striatal D2 receptors, with a higher sensitivity for males. No alterations in spatial learning and memory was observed, but impairments of motor activity, increased anxiety (open fiel measurment), which are other symptoms of autism spectrum disorder.

Franco et al., 2006. Lactational exposure of mice to methylmercury in drinking water (10 mg/L). Analysis at weaning revealed only impairment in motor performances.

Franco et al., 2007. Lactational exposure of mice with mercury chloride (0.5 and 1.5 mg/kg, i.p. injection once a day).. At weaning , animals exhibited an increased level of mercury in cerebellum associated with motor deficit.

Cardenas et al., 2017 showed that maternal red blood cell mercury of 3.8 ng/g was associated to increased DNA methylation of PON1 in umbilical cord blood only in male and observed deficit in cognitive performances, such as visual motor ability, vocabiary and verbal intellgence.

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List of Non Adjacent Key Event Relationships

Relationship: 3299: Dendritic spine abnormality leads to Impairment, Learning and memory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	non-adjacent	High	High

Relationship: 3300: Loss of drebrin leads to Impairment, Learning and memory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Human, rat, mouse	Human, rat, mouse	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	Not Specified

Key Event Relationship Description

Loss of drebrin disrupts dendritic spine morphology and impairs synaptic plasticity, leading to deficits in learning and memory. Experimental evidence demonstrates that drebrin reduction, achieved either through genetic deletion (up to 80–90%) or antisense knockdown, results in decreased spine density and increased spine length in hippocampal neurons. These structural abnormalities coincide with functional impairments, including reduced hippocampal synaptic plasticity, disrupted regulation of NMDA receptors or associated receptor complexes, and enhanced mGluR5-dependent long-term depression. Animal studies consistently link these molecular and cellular changes to impaired fear learning, spatial memory, and broader cognitive functions.

Implicitly, these findings suggest that drebrin is essential for maintaining proper neuronal circuitry and synaptic integrity, and that its depletion may broadly compromise neuronal network functionality and cognitive processing, even though such broader implications are not explicitly detailed in the original description of key events.

Relationship: 2808: Dysfunctional synapses leads to Impairment, Learning and memory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	non-adjacent	Moderate	Moderate

Relationship: 3506: Binding of agonist, Ionotropic glutamate receptors leads to Loss of drebrin

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of chemicals to ionotropic glutamate receptors leads to impairment of learning and memory via loss of drebrin from dendritic spines of neurons	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human, mouse, rat	human, mouse, rat	High	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Mixed	Not Specified

Key Event Relationship Description

Glutamate stimulation (100 µM;10 min) of cortical and hippocampal cultured neurons induced disappearance of drebrin immunostaining from dendritic spines but led to appearance of drebrin immunostaining in dendritic shafts and somata. The glutamate-induced shift of drebrin immunostaining was blocked by an NMDA receptor antagonist. Immunoblot analyses showed that both the total and the cytosolic drebrin remained unchanged and revealed that the drebrin shift was not due to drebrin degradation for the stimulation condition.