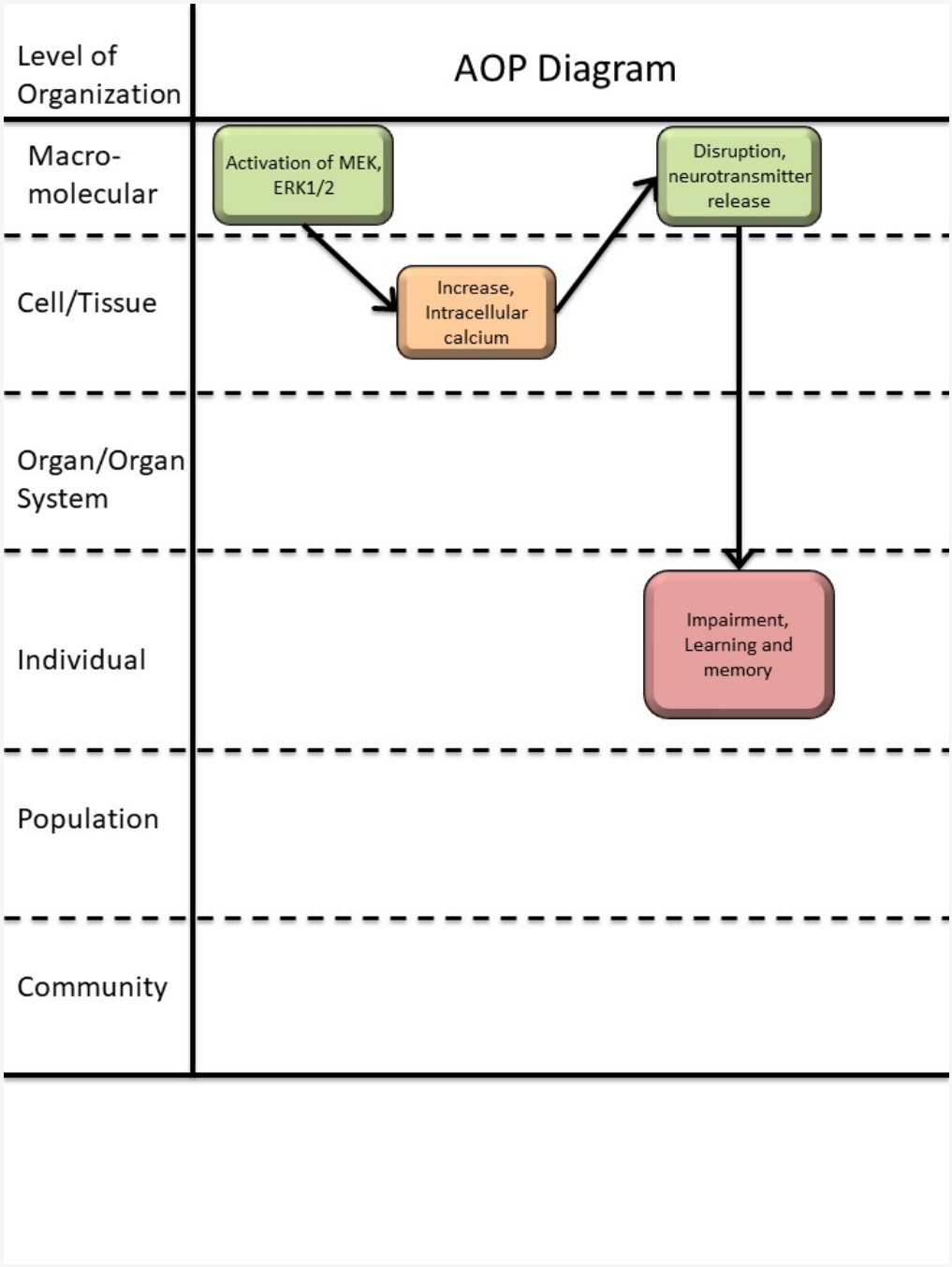
AOP ID and Title:

AOP 499: Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release
Short Title: MEK-ERK1/2 activation leading to deficits in learning and cognition

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite			

Abstract

Metal mixture activation of ERK1/2 and JNK1/2 in astrocytes leads to increased C^{a2+} release (Asit Rai and others 2010). Alterations to calcium, an essential nutrient which is required in multiple cellular and physiological functions, such as cell adhesion, signal transduction, and neurotransmission can be expected to have downstream effects in those functions (Antonio et al., 2002). Changes in neurotransmission can then lead to changes in learning and cognitition (Neal and Guilarte 2010).

MEK-ERK1/2 is important in understanding uptake of metals into the brain and its relationship to deficits in learning and cognition from exposure to metals commonly detected at Superfund sites including lead, cadmium, manganese, and arsenic. Current risk assessment guidance dictates a largely chemical-by-chemical evaluation of exposures and risks, which fails to adequately address potential interactions with other chemicals, nonchemical stressors, and genetic factors. Cumulative risk assessment methods and approaches are evolving to meet regulatory needs, (MacDonell et al., 2013; Backhaus and Faust 2012; IPCS Workshop 2009) but significant challenges remain. As our understanding of complex exposures and interactions continues to grow, synthesis and integration across disciplines and studies focused on different aspects of the environmental fate–exposure–toxicology–health outcome continuum are required to assess the likelihood of adverse effects and to support cumulative risk assessment. Environmental exposures are virtually always to complex mixtures (Katherine von Stackelberg et al., 2015).

Background

An examination of neurodevelopmental disorders and subclinical effects using multi-domain global neurodevelopment assessments is warranted as they can have profound population level implications. In the context of neurotoxicity, neurodevelopmental pathways in the developing human brain are not fully understood (Schubert et al., 2015; Bal-Price et al., 2015) although there are a number of commonly observed phenomena which may take part in those pathways e.g. changes in intracellular calcium, ROS generation, apoptosis, and neurotransmitter disruption. This AOP highlights a specific set of response-response relationships using a subset of those commonly observed phenonema related to metals and metal mixture exposures leading to deficits in learning and cognition.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	2146	Activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase 1/2	Activation of MEK, ERK1/2
	KE	1339	Increase, intracellular calcium	Increase, intracellular calcium
	KE	2151	Disruption, neurotransmitter release	Disruption, neurotransmitter release
	AO	341	Impairment, Learning and memory	Impairment, Learning and memory

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase 1/2	adjacent	Increase, intracellular calcium	Not Specified	Not Specified
		Disruption,		

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increase, intracellular calcium	adjacent	neurotransmitter release	Not Specified	Not Specified
Disruption, neurotransmitter release	adjacent	Impairment, Learning and memory	Not Specified	Not Specified

Stressors

Name	Evidence
Lead	
Arsenic	
Cadmium	
Manganese	
Heavy metals (cadmium, lead, copper, iron, nickel)	

Overall Assessment of the AOP

1. Support for Biological Plausibility of KERs	Defining Question	High (Strong)	Moderate	Low (Weak)
	Is there a mechanistic relationship between KE_{up} and KE_{down} consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs , but the structural or functional relationship between them is not understood.
Relationship 2942: Activation of MEK, ERK1/2 (2146) leads to Increase, intracellular calcium (1339)	**Moderate** Empirical evidence indicates a complex relationship between MEK, ERK1/2 activation and inhibition and Ca2+ response including Ca2+ feeding back into a ERK1/2 activation. This relationship appears to vary across species and cell type.			
Relationship 2954: Increase, intracellular calcium (1339) leads to Disruption, neurotransmitter release (2151)	**Strong** Intracellular calcium regulation is broadly known as being an important aspect of a number of processes in a variety of cells and is particularly critical in nerve cell terminals where it mediates transmitter release.			
Relationship 2955: Disruption, neurotransmitter release (2151) leads to Impairment, Learning and memory (341)	**Strong** The role of various neurotransmitters and receptors in cognitive function and memory formation are well studied.			

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence

Sex	Evidence
Unspecific	Moderate

Life Stage

Life stages applicable to this AOP encompass the full life cycle. Many of the key events are measured in pregnant females with the adverse outcome (impairment, learning and memory) measured at all life stages.

Taxonomic Applicability

Most evidence for this AOP is derived from rodents and humans where rodents were selected with their ability to model human responses.

Sex Applicability

This AOP is applicable to all sexes.

Essentiality of the Key Events

2. Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
MIE 2146:Activation of MEK, ERK1/2	**Moderate** MEK, ERK1/2 activation is fundamental in delivering signals which regulate the cell cycle, proliferation, differentiation, adhesion, and more. Disruptions in this activation have wide reaching effects however, there is evidence that downstream KEs can also activate this KE.			
KE 1339: Increase, intracellular calcium	**High** Calcium, as a primary intracellular messenger in neurons and regulator of cell responses to stress has been shown to directly affect neurotransmitter release with manipulation.			
KE 2151: Disruption, neurotransmitter release	**High** Neurotransmitter receptor blocking experiments have shown to directly impair learning and memory tasks in rodents.			
AO 341: Impairment, Learning and memory	**N/A**			
AOP 499	**High/Moderate** There is direct evidence contained KER 2955.			

Weight of Evidence Summary

3. Empirical Support for KERs	Defining Questions	High (Strong)	Moderate	Low (Weak)
	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup > than that for KEdown? Inconsistencies?	if there is dependent change in both events following exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance) and no or few data gaps or conflicting data	if there is demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with the expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	if there are limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all), and/or lacking evidence of temporal or dose-response concordance, or identification of significant inconsistencies in empirical support across taxa and species that don't align with the expected pattern for the hypothesized AOP

Relationship 2942: Activation of MEK, ERK1/2 (2146) leads to Increase, intracellular calcium (1339)	**Moderate** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure.
Relationship 2954: Increase, intracellular calcium (1339) leads to Disruption, neurotransmitter release (2151)	**Moderate** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure.
Relationship 2955: Disruption, neurotransmitter release (2151) leads to Impairment, Learning and memory (341)	**Moderate** The evidence collection strategy for this AOP focused mainly on metal and metal mixture exposures, of which, there were many that showed dependent change in both these events following exposure.

Considerations for Potential Applications of the AOP (optional)

Developmental neurotoxicity (DNT) is an adverse outcome of concern to multiple regulatory agencies. In vitro screening assays for MEK-ERK1/2 activation would not be recommended as a direct alternative or replacement to established DNT assays like OECD Test No. 426 (OECD 2007). However, detection of MEK-ERK1/2 activation in neuronal cell types may be used to prioritize chemicals with potential to elicit neurotoxicity and flag them for testing in ortogonal assays for evaluating DNT, including proposed alternative test methods (Bal-Price et al. 2018; Crofton et al 2022).

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Appendix 1

List of MIEs in this AOP

Event: 2146: Activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase 1/2

Short Name: Activation of MEK, ERK1/2

Key Event Component

Process	Object	Action
kinase activity	astrocyte	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	MolecularInitiatingEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

astrocyte

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Mixed	Moderate

Key Event Description

ERK1 and ERK2 are proteins of 43 and 41 kDa that are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates (Boulton et al., 1990; 1991). The two phosphoacceptor sites, tyrosine and threonine, which are phosphorylated to activate the kinases, are separated by a glutamate residue in both ERK1 and ERK2 to give the motif TEY in the activation loop (Payne et al., 1991). Both are ubiquitously expressed, although their relative abundance in tissues is variable. For example, in many immune cells ERK2 is the predominant species, while in several cells of neuroendocrine origin they may be equally expressed (Gray Pearson and others 2001). They are stimulated to some extent by a vast number of ligands and cellular perturbations, with some cell type specificity (Lewis et al., 1998). In fibroblasts (the cell type in which the generalizations about their behavior and functions have been developed) they are activated by serum, growth factors, cytokines, certain stresses, ligands for G protein-coupled receptors (GPCRs), and transforming agents, to name a few (Gray Pearson and others 2001). They are highly expressed in postmitotic neurons and other highly differentiated cells (Boulton et al., 1991). In these cells they are often involved in adaptive responses such as long-term potentiation (English and Sweatt 1996; Atkins et al., 1998; Rossi-Arnaud et al., 1997).

How it is Measured or Detected

Western blotting and immunoblotting.

References

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List of Key Events in the AOP

Event: 1339: Increase, intracellular calcium

Short Name: Increase, intracellular calcium

Key Event Component

Process	Object	Action
calcium amount	calcium(2+)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:214 - Network of SSRIs (selective serotonin reuptake inhibitors)	KeyEvent
Aop:226 - SSRI (Selective serotonin reuptake inhibitor) to hypertension	KeyEvent
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	KeyEvent
Aop:500 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate
Birth to < 1 month	Moderate

Sex Applicability

Sex	Evidence
Mixed	Moderate

Key Event Description

Calcium is arguably the most versatile and important intracellular messenger in neurons (Berridge et al., 2000). Interestingly, although calcium may often promote neuronal death, it can also activate pathways that promote survival. For example, calcium can promote survival through a pathway involving activation of protein kinase B (PKB/Akt) by calcium/calmodulin-dependent protein kinase (Yano et al., 1998). Calcium is a prominent regulator of cellular responses to stress, activating transcription through the cyclic-AMP response element-binding protein (CREB), which can promote neuron survival in experimental models of developmental cell death (Hu et al., 1999). Calcium can also activate a rapid neuroprotective signalling pathway in which the calcium-activated actin-severing protein gelsolin induces actin depolymerization, resulting in suppression of calcium influx through membrane NMDA (N-methyl-d-aspartate) receptors and voltage-dependent calcium channels (Furukawa et al., 1997). This may occur through intermediary actin-binding proteins that interact with NMDA receptor and calcium channel proteins. Finally, signals such as calcium and secreted amyloid precursor protein-α (sAPP-α), which increase cyclic GMP production, can induce activation of potassium channels and the transcription factor NF-κB, and thereby increase resistance of neurons to excitotoxic apoptosis (Furukawa et al., 1996).

How it is Measured or Detected

An increase in $[Ca^{2+}]_i$ was measured using Fluo3 AM as an indicator dye after the addition of metals (single or in mixture) to the culture wells following an optimized protocol (Arey et al., 2022). The fluorescent signals were read by fluorescence imaging plate reader Synergy HT (BioTek, Winooski, VT) (Rai and others 2010).

Briefly, Ca2+ levels in human astrocytes were monitored by fluorescence microscopy using the Ca2+ indicator fluo-4. Slices were incubated with fluo-4-AM (2–5 µL of 2 mM dye were dropped over the tissue, attaining a final concentration of 2–10 µM and 0.01% of pluronic) and Sulforhodamine 101 (100 µM) for 30–60 min at room temperature (Navarrete and others 2013). In these conditions, most of the Fluo-4-loaded cells were astrocytes as indicated by their SR101 staining (Nimmerjahn et al., 2004; Dombeck et al., 2007; Kafitz et al., 2008; Takata and Hirase 2008), and confirmed in some cases by their electrophysiological properties. Astrocytes were imaged with an Olympus FV300 laser-scanning confocal microscope or a CCD camera (Retiga EX) attached to the Olympus BX50WI microscope (Navarrete and others 2013).

Diversity of endogenous Ca2+ activity in a mature hippocampal astrocyte in situ: Ca2+ signals in cell body and processes are different. (A) Cumulative Ca2+ activity recorded in an astrocyte over a 165 s period revealed by the calcium indicator Fluo4-AM. The visible boundaries of the astrocyte are shown in white. Note the different intensities of spatially-
confined local activity in the astrocyte cell body (s), primary process (p1) stemming from the soma and secondary processes (p2) branching from a primary process. Intensity of the
normalized cumulative activity is expressed in arbitrary units (a.u.) and shown in pseudocolour, from dark (lowest) to white (highest). (B) Frequency map of the Ca2+ activity in the astrocyte during the 165 s period as in A. Activity is measured in individual pixels, expressed in mHz and color-coded from black (never active) to dark red (frequently active). Most of the activity is within the white boundaries and the most frequently active pixels are in defined small regions (arrowheads) of the primary and secondary processes (30 mHz), whereas pixels of the soma are less active (~10 mHz) (Volterra et al., 2014).

Free intracellular calcium ions were measured using the fluorescent calcium indicator FLUO-3/AM (Molecular probes, Eugene, OR, USA). Cells (4×10^4 cells/cm^2) were seeded in 24-well plates for 24 h to reach 60%–70%, and then treated for 24 h with As(III) (0.5 and 1 mg/l), or coexposed to As(III) (1 mg/l) and F (2.5, 5, and 10 mg/l). After treatment, supernatant was collected and combined with trypsinized cells. Pelleted samples were resuspended in 500 µl of FLUO-3/AM (4 µmol/l) and incubated at 37 °C for 30 min. After centrifugation, cells were washed with HBSS (Hank's Buffered Salt Solution, Sigma), made up to 400 µl with HBSS and analyzed by flow cytometry. The signal from FLUO-3/AM bound to Ca^{2+} was recorded using the Fl-1 channel (Rocha et al., 2011).

Fluo-4/AM was used as an intracellular free Ca^{2+} fluorescent probe to analyze $[Ca^{2+}]_i$ in Cd-exposed cerebral cortical neurons. In short, the harvested cells were incubated with Fluo-4/AM (5 µmol/L final concentration) for 30 min at 37°C in the dark, washed with PBS, and analyzed on a BD-FACS Aria flow cytometry. Intracellular $[Ca^{2+}]_i$ levels were represented by fluorescent intensity. Fluorescent intensity was recorded by excitation at 494 nm and emission at 516 nm. The data were analyzed by Cell Quest program (Becton Dickinson), and the mean fluorescence intensity was obtained by histogram statistics (Yuan et al., 2013).

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Event: 2151: Disruption, neurotransmitter release

Short Name: Disruption, neurotransmitter release

Key Event Component

Process	Object	Action
signaling	neurotransmitter	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	KeyEvent
Aop:471 - Various neuronal effects induced by elavl3, sox10, and mbp	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

neuron

Organ term

Organ term

brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	Moderate
Adult	Moderate

Sex Applicability

Sex	Evidence
Mixed	Moderate

Key Event Description

Any of various neurotransmitters or indicators of neurotransmission.

How it is Measured or Detected

Weighed brain tissues were homogenized in a Potter-Elvehjem type A homogenizer with a teflon pestle using cold acidified n-butanol. The biogenic amines were extracted and estimated according to the procedure of Sadavongvivad (1970). The recovery experiments were done simultaneously. Recoveries for different standards were 92 + 3% for dopamine (DA), 80+ 5% for norepinephrine (NE) and 90 + 5% for 5-hydroxytryptamine (5-HT). Fluorescence was measured in a Aminco SPF-500 spectrofluorometer (Chandra et al., 1981).

BDNF quantitative real-time PCR. Hippocampal neuronal cultures were exposed to normal bath solution or 1.0 or 2.0μM Pb2+ for 5 days, and subsequently RNA was harvested according to manufacturer's instructions (RNeasy; Qiagen), quantified by reading the absorbance at 260 nm, and converted to complementary DNA (cDNA) using 1 μg RNA according to manufacturer's instructions (High Capacity Reverse Transcription Kit 4368814; Applied Biosystems). Quantitative real-time PCR (q-rtPCR) was performed in triplicate using TaqMan Gene Expression Assays (Applied Biosystems) with 50 ng cDNA using the following probes: Actin (Rat, Rn00667869_m1; Applied Biosystems) and BDNF exon I, exon II, exon IV, and exon IX (Applied Biosystems). Data were analyzed as previously described (Livak and Schmittgen, 2001), and results were expressed as fold change in gene expression relative to control (Stansfield and others 2012).

BDNF release via ELISA. Sandwich ELISAs were performed on DIV12 cell culture media using the BDNF Emax ImmunoAssay System kit (Promega, Madison, WI) according to the manufacturer's instructions. BDNF content was interpolated from standard curve runs for each plate (linear range of 7.8–500 pg/ml). BDNF protein content was divided by total protein for each sample to determine the number of picograms of peptide per microgram of total protein (Stansfield and others 2012).

In vivo microdialysis is a well-established method for monitoring the extracellular levels of neurotransmitters in the CNS. This technique has been used extensively in neuroscience for almost 30 years (Westerink 1995; Ungerstedt 1991; Robinson 1991; Benveniste 1989; Benveniste and Huttemeier 1990; Di Chiara 1990). Microdialysis allows online estimates of neurotransmitters in living animals and is a suitable method for monitoring the extracellular levels of neurotransmitters during local administration of pharmacological agents (Hammarlund-Udenaes 2000). Older alternative *in vivo* methods for the study of neurotransmitter release are the push–pull technique used in the brain, (Singewald and Philippu 1998) spinal cord, (Zachariou and Goldstein 1997) and intrathecal space (Yaksh and Tyce 1980).

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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:442 - Binding to voltage gate sodium channels during development leads to cognitive impairment	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 socio-economic burden through reduced IQ and non-cholinergic mechanisms	AdverseOutcome
Aop:499 - Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	AdverseOutcome

AOP ID and Name	Event Type
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

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
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

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 behaviour. On the other hand, non-associative learning can be defined as an alteration in the behavioural response that occurs over time in response to a single type of stimulus. Habituation and sensitization are some examples of non-associative learning.

The memory formation requires acquisition, retention and retrieval of information in the brain, which is characterised 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 neuronal 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 behavioural 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.

1) 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).

2) 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).

3) 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).

4) 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).

5) 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:

1) 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).

2) 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).

3) 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).

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

5) 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).

6) 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).

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

8. Comprehensive developmental inventory for infants and toddlers (CDIIT). The CDIIT was designed and standardized in 1996, and it measures the global, cognitive, language, motor, gross motor, fine motor, social, self-help and behavioral developmental status of children from 3 to 71 months old (Wang et al., 1998).

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 behaviour. 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: 2942: Activation of MEK, ERK1/2 leads to Increase, intracellular calcium

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	adjacent	Not Specified	Not Specified
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via ROS and apoptosis	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	Moderate

Life Stage	Evidence
1 to < 3 months	Moderate
Pregnancy	Moderate

Sex Applicability

Sex	Evidence
Female	Moderate
Mixed	Moderate

Key Event Relationship Description

Astrocytes are networked together by a series of gap junctions permitting to propagate Ca^{2+} waves through the linked network (Lobsiger and Cleveland 2007), and Ca2+-mediated intercellular communication is a mechanism by which astrocytes communicate with each other and modulate the activity of adjacent cells (Verderio et al., 2001). Metal mixture (MM) induced alteration in astrocyte morphology may influence [Ca^{2+}]i (Barres et al., 1989); in contrast, an increase in $[Ca^{2+}]_i$ may also play a key role in altering astrocyte cytoskeleton, affecting the glia-neuron interaction (Shelton et al., 2000).

Inhibition of GFAP immunoreactivity by MM in developing brain appears to be caused by astrocyte apoptosis. In primary cultures of astrocytes, our data show that MM synergistically induced apoptosis (Rai and others 2010). This was manifested by the activation of MEK/ERK, followed by the activation of JNK pathways, which then enhanced intracellular Ca2+ levels and subsequently ROS generation.

Evidence Supporting this KER

Empirical Evidence

We treated the astrocytes with a metal-mixture (MM) of arsenic, cadmium, and lead and observed that the MM triggered $[Ca^{2+}]_i$ release (Rai and others 2010). The $[Ca2+]_i$ release reached its peak after 30 min of MM treatment. Similarly, MM triggered ROS generation, and the ROS generation reached its peak after 1 h of MM treatment. To investigate whether the $[Ca^{2+}]_i$ release was ROS, ERK1/2, or JNK1/2 –dependent, we incubated the MM-treated astrocytes with an antioxidant (a-tocopherol, 200 lg/ml), PD98059 (10lM), or SP600125 (10lM). a-Tocopherol itself was nontoxic. We observed that PD98059 (10lM) or SP600125 (10lM) suppressed $[Ca^{2+}]_i$ release, but a-tocopherol (200 lg/ml) did not. This suggested that $[Ca^{2+}]_i$ release in MM-treated astrocytes was ERK1/2 and JNK1/2 dependent (Rai and others 2010).

Yael and Breitbart (2015) demonstrated for the first time that mouse sperm ERK1/2 is activated upon ZP addition, and that ERK1/2 mediates the elevation of intracellular Ca2+ in the sperm cell prior to the occurrence of the acrosome reaction. The fact that the acrosome reaction, induced by the Ca^{2+}-ionophore A23187, was not inhibited by U0126 suggests that ERK1/2 mediates the acrosome reaction by activating Ca2+ transport into the cell. Direct determination of intracellular [Ca^{2+}] revealed that Ca2+ influx induced by EGF or ZP was completely blocked by U0126. Thus, it has been established that the increase in ERK1/2 phosphorylation/activation in response to ZP or by activation of the EGF receptor (EGFR) by EGF, is a key event for intracellular Ca^{2+} elevation and the subsequent occurrence of the acrosome reaction (Jaldety et al., 2015).

To examine the relationship between Ca^{2+} and Erk1/2 signaling, Levin and Borodinsky (2022) inhibited Mek1/2 with PD0325901 and found that this prevents the injury-induced increase in Ca^{2+} activity in cells lateral to the axial musculature across the entire 800 µm-wide region measured. This suggests that injury-induced Erk1/2 activation recruits Ca^{2+} activity to promote regeneration of the larval tail. Consistent with recruitment of Ca^{2+} activity across a wide region of tail, activated Erk1/2 is also present in at least the posterior 800 µm of stump (Levin et al., 2022). However, unlike Ca^{2+} activity, Erk1/2 signaling at 20 mpa is activated in a gradient. This could mean that even the lowest level of Erk1/2 signal measured in 800 µm of amputated tail is sufficient to induce the Ca^{2+} response, or that a signal is propagated anteriorly from the cells adjacent to the amputation where injury induces high Erk1/2 activation (Levin et al., 2022).

Quantitative Understanding of the Linkage

Time-scale

Exposures were conducted for 2 min, 5 min, 10 min, 30 min, 1 h, 2 h, and 24 h. The $[Ca2+]_i$ release reached its peak after 30 min of MM treatment (Rai and others 2010).

Known Feedforward/Feedback loops influencing this KER

The activity of many protein kinases is modulated by Ca2+ and/or Ca2+/calmodulin either directly (PKC, CaM kinase II) or indirectly (PKA via stimulation of adenylyl cyclase and phosphodiesterase by Ca2+/calmodulin) (Kern et al., 1995). Therefore, the effects of Ca2+ and protein kinases on cytoskeletal proteins and neurite initiation are likely to be mediated, at least in part, by changes in protein phosphorylation (Kern et al., 1995).

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Relationship: 2954: Increase, intracellular calcium leads to Disruption, neurotransmitter release

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Female	Moderate
Mixed	Moderate

Key Event Relationship Description

While intracellular Ca regulation is an important aspect of a number of processes in a variety of cells, it is particularly critical in nerve cell terminals where Ca mediates transmitter release (Augustine et al., 1987). Many synaptic connections during brain development involve calcium signaling, which directs structural as well as functional adaptation in neurons (Lohmann 2009; Michaelson and Lohmann 2010) and astrocytes (Navarette et al., 2013) to establish synaptic selectivity in the developing brain (Katherine von Stackelberg 2015). While astrocytes have long been known to support neuronal signaling, there is increasing evidence that astrocytes detect synaptic activity and engage in reciprocal signaling with neurons, again based on variations in intracellular Ca^{2+} (Volterra et al., 2014; Barkera and Ullian 2008).

Evidence Supporting this KER

Biological Plausibility

Lead (1-30 uM) was also observed to induce a concentration-dependent release of dopamine from striatal synaptosomes under conditions of spontaneous release (Minnema et al., 1986). Similar lead induced neurotransmitter release has been demonstrated for acetylcholine at the neuromuscular junction, as reflected by increase miniature-end-plate potentials (Cooper and Manalis 1983), and in cortical synaptosomes (Suszkiw et al., 1984). Although the mechanisms by which lead induces transmitter release are unresolved, the increased release may result from an increase in intrasynaptosomal free calcium which has been shown to increase release (Katz 1969).

Empirical Evidence

Lead could act to increase spontaneous transmitter release by increasing the intraneuronal ionized Ca concentration (Kolton and Yarri 1982). One means by which the intraneuronal free Ca could be elevated is by inhibition of Ca extrusion; specifically, inhibition of the Mg2+-dependent Ca-ATPase (Minnema et al., 1988). The extrusion of Ca by Ca-ATPase at the plasma membrane is the dominant means by which the intraneuronal Ca concentration is maintained during “resting” conditions (Snelling and Nicholls 1985). Although Pb has been reported to be a weak inhibitor of this enzyme (Thompson and Nechay 1981), the Pb-induced increase in 45Ca efflux observed in the current study (Minnema et al., 1988) would not be expected if Ca-ATPase inhibition is the mechanism by which Pb increases transmitter release. The similar concentration/release effects and temporal relationships between transmitter release and 45Ca efflux suggest that Pb may displace bound Ca from intraneuronal Ca sources (Minnema et al., 1988). The slight temporal differences in onset and peak effects (i.e., the effect of Pb on transmitter release precedes its effect on 45Ca efflux) are consistent with the view that Pb increases the intraneuronal ionized Ca concentration, which would first interact at the intraneuronal site mediating transmitter release, and subsequently this Ca would be extruded from the nerve ending (Minnema et al., 1988).

We next investigated the consequences of astrocyte Ca^{2+} signal on human neurons. In hippocampal slices, local application of ATP evoked astrocyte Ca2+ elevations that propagated as a wave throughout the Stratum radiatum reaching the Stratum pyramidale, and then evoking Ca^{2+} elevations in pyramidal neurons after a conspicuous delay from the initial astrocyte Ca^{2+} elevations, suggesting that astrocyte Ca^{2+} stimulates the release of gliotransmitters that acting on transmitter receptors affect the intracellular Ca^{2+} levels in human neurons (Navarette et al., 2013).

Local application of ATP, which elevated Ca^{2+} levels in astrocytes, also increased the frequency of slow inward currents (SIC) in both hippocampal and cortical neurons. While SIC frequency was insensitive to TTX (n = 3 neurons), SICs were abolished by 50 µM AP5, indicating that they were independent of action potential-evoked neurotransmitter release and that they were mediated by NMDARs. Therefore, in agreement with compelling evidence obtained in rodents (Parri et al., 2001; Fellin, Tommaso, et al. 2004; Gertrudis and Araque 2005; Navarrete et al., 2008; Shigetomi, Eiji, et al. 2008; Bardoni, Rita, et al., 2010; Sasaki, Takuya, et al. 2011), Ca^{2+} elevations in human astrocytes stimulate the release of glutamate that activates NMDARs in neurons, indicating the existence of gliotransmission and astrocyte-to-neuron communication in human brain tissue (Navarette et al., 2013).

Uncertainties and Inconsistencies

Synaptotagmin I (Syt) is a Ca^{2+} -sensing protein found in neurotransmitter vesicles and is responsible for promoting vesicular fusion in the presence of Ca^{2+} signaling (Chicka et al., 2008). Pb^{2+} bound Syt with 1000-fold higher affinity than Ca^{2+}, which may prevent detection of Ca^{2+} signaling essential to neurotransmission (Bouton et al., 2001). Although Pb^{2+} exposure did not affect Syt protein expression in cultured hippocampal neurons (Neal et al., 2010), it is possible that Pb^{2+} may interfere with the Ca^{2+}-sensing ability of Syt in neurons, thus masking the cellular signal for Ca^{2+}-dependent vesicular release (Neal and Guilarte 2010).

Pb^{2+} interactions with Syt may be related to the ability of Pb^{2+} to mimic Ca^{2+} (Neal and Guilarte 2010). Pb^{2+} has an ionic radius of 1.2 Å, which is similar to the ionic radius of Ca^{2+} (0.99 Å) (Chao et al., 1984; Garza et al., 2006). The positive charges and high electronegativity (2.33 on the Pauling scale) of Pb^{2+} may allow it to interact with the same residues on Ca^{2+} binding sites that interact with Ca^{2+} ions (Garza et al., 2006). Pb^{2+} has been shown to interact with several neuronal intracellular Ca^{2+}-binding proteins in addition to Syt (described above), such as the Ca^{2+}-binding protein calmodulin (CaM) (Chao et al., 1984; Habermann et al., 1983; Kern et al., 2000), the CaM/Ca^{2+}-dependent phosphatase calcineurin (Kern and Audesirk 2000), CaMKII (Toscano et al., 2005), and protein kinase C (Simons 1993; Sun et al., 1999; Toscano and Schanne 2000; Long et al., 1994), suggesting that Ca^{2+} mimicry may be a common characteristic of Pb^{2+} toxicity (Bressler et al., 1999; Marchetti 2003; Richardt et al., 1986). Thus, the ability of Pb^{2+} to mimic Ca^{2+} may interfere with normal synaptic signaling events (Neal and Guilarte 2010).

Another hypothesis regarding the disruption of neurotransmission is that Pb^{2+} may interfere with Ca^{2+} signals by inhibiting Ca^{2+} channels (Xiao et al., 2006; Braga et al., 1999; $^{35)}$. Neurotransmission relies on the influx of Ca^{2+} from P/Q-, N-, and to some extent R-type voltage-gated Ca^{2+} channels (VGCCs) (Xu et al., 2007). Pb^{2+} has been shown to inhibit VGCCs in recombinant systems with high affinity (Peng et al., 2002). Furthermore, removal of extracellular Ca^{2+} resulted in identical effects on IPSC frequency as Pb^{2+} exposure, suggesting that the Pb^{2+}-induced inhibition of IPSC frequency is via reduction of Ca^{2+} influx through VGCCs (Xiao et al., 2006). Inhibition of presynaptic VGCCs may prevent the necessary rise in internal Ca^{2+} required for fast, Ca^{2+}-dependent vesicular release, thus interfering with neurotransmission (Neal and Guilarte 2010).

Cadmium may block the influx of Ca^{2+} through membrane channels into the nerve terminal following the action potential, these decrease in calcium influx caused by Cd would be associated with an altered transmitter release (Antonio et al., 1999).

Quantitative Understanding of the Linkage

Time-scale

Calcium efflux and induced spontaneous transmitter release occur on a seconds to minutes time-scale (Minnema et al., 1988).

Known Feedforward/Feedback loops influencing this KER

It has been clear for quite some time that influx of calcium at the synapse mediates synaptic plasticity in adult as well as developing neurons (Malenka et al., 1988). Despite this long-standing appreciation of the importance of calcium signaling for synaptic plasticity, it is virtually unknown what the properties of calcium transients are that determine whether a synapse becomes potentiated or depressed (Malenka and Bear 2004). Some models suggest that moderate increases in calcium may activate primarily phosphatases (e.g., calcineurin and protein phosphatase-1) that in turn facilitate synaptic depression (Mansuy and Shenolikar 2006). In contrast, the activation of kinases (e.g., calcium/calmodulin-dependent protein kinase II, CaMKII) by high-amplitude calcium transients may favor potentiation (Lisman et al., 2002). This is in fact an interesting parallel to the regulation of attractive vs. repulsive axon guidance by calcium: larger calcium transients can activate CaMKII and induce turns toward the side of calcium elevation, whereas smaller calcium increases activate the phosphatases calcineurin and phosphatase-1 and trigger repulsive turns (Wen et al., 2004; Zheng and Poo 2007).

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Relationship: 2955: Disruption, neurotransmitter release leads to Impairment, Learning and memory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Activation of MEK-ERK1/2 leads to deficits in learning and cognition via disrupted neurotransmitter release	adjacent	Not Specified	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Neurotransmitters and their receptors are essential for brain functioning, learning, and memory. Catecholamines, including dopamine and norepinephrine, are the main neurotransmitters that mediate a variety of central nervous system (CNS) functions, such as motor control, cognition, emotion, memory processing, and endocrine modulation, determined by recent molecular genetic approaches in mice (Handra et al., 2019).

Evidence Supporting this KER

Biological Plausibility

The N-methyl-D-aspartate receptor (NMDAR) plays an essential role in hippocampus-mediated learning and memory, based on studies showing that intra-ventricular administration of an NMDAR antagonist (aminophosphonovaleric acid (APV)) in rats resulted in spatial learning impairments similar to those encountered with hippocampal lesions (Morris et al., 1982; Morris et al., 1986).

Memory acquisition is considered to involve both short-term changes in electrical properties and long-term structural alterations in synapses. Short-term changes may include LTP and LTD whereas long-term morphological alterations may involve synaptogenesis and neuropil growth (Burns and Augustine 1995; Edwards 1995). Since BDNF significantly modulates both forms of synaptic changes and the expression is upregulated during memory acquisition, as described above, it may play a role in learning and

memory (Lo DC 1995; Thoenen 1995; McAllister et al., 1999).

Cortical acetylcholine release increases (1) during acquisition but not during recall of a rewarded operant behavior (Orsetti et al., 1996), (2) during acquisition of operant tasks when demands on attentional processing are high (Muir et al., 1996), (3) during conditioned taste aversion (Miranda et al., 2003), and (4) during performance of visual attentional tasks (Himmelheber et al., 2001). It has been also related to attentional effort (Himmelheber et al., 2001). Furthermore, in the hippocampus, ACh release increases during the performance of a learned spatial memory task (Ragozzino et al., 1996; Stancampiano R, et al., 1999) and the increase is positively correlated to performance improvement during task learning (Fadda et al., 2000), showing that cholinergic neurons are modified functionally during learning and become progressively more active. Also, the initial use of a place strategy coincided with an immediate increase in hippocampus ACh release (Chang and Gold 2003). Furthermore, as rewarded spontaneous alternation testing progressed, a switch to a repetitive response strategy accompanied an increase in striatum ACh release (Pych JC et al., 2005).

The release of acetylcholine in different brain areas appears to be involved in processes of attention (Marrosu et al., 1995), detection of novelty or saliency (Baxter et al., 1999), and during the consolidation of different types of long-term memory (Power 2004; McIntyre et al., 2003; Hasselmo 1999).

Empirical Evidence

Miranda 2007 reviews many studies which demonstrate the activation of neurotransmitters such as glutamate, noradrenaline, and dopamine in several types of learning and during several stages of memory formation. The results of innumerable studies indicate that during memory formation different regions of the brain act in coordinated fashion through different neurotransmission systems (Miranda 2007).

Targeted knockout of the NMDAR in the hippocampus impairs spatial learning (Neal and Guilarte 2010), lending further support to the role of the NMDAR in hippocampus-mediated learning processes.

A neurotransmitter system that has been previously linked with the cognitive functions is the glutamate NMDA receptor system (May Simera and Levin 2003; Li et al., 2013). In 1991, Izquierdo, with the help of NMDA receptor antagonists (which impaired spatial working memory), concluded that if repeatedly stimulated, this system can regulate cognition (Izquierdo 1991). What is more, it was observed that blocking the NMDA receptor induces a resembling degree of memory impairment as the excision of the hippocampus (Lupușoru et al., 2017).

Learning-induced increases of ACh in the hippocampus and cortex have two important characteristics that strongly suggest that these increases are involved in memory consolidation (Power et al., 2003). First, the ACh increases induced during acquisition persist for at least 15 min after the end of the task (Orsetti et al., 1996; Ragozzino et al., 1996; Kopf et al., 2001; Miranda and Bermúdez-Rattoni 1999; Toumane et al., 1988). It is well established that during this posttraining period memory consolidation is strongly influenced by endogenous hormones and is highly susceptible to disruption and modulation by pharmacological interventions (McGaugh and Izquierdo 2000; McGaugh 2000). Furthermore, the persistence of the ACh levels in the hippocampus and cortex is correlated with the duration of these structures' involvement in memory consolidation (Power et al., 2003).

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