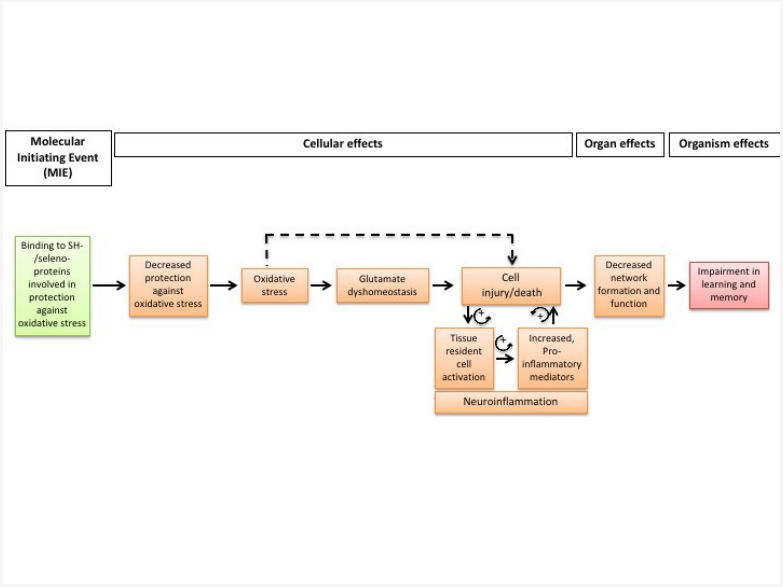
AOP ID and Title:

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
Short Title: Oxidative stress and Developmental impairment in learning and memory

Graphical Representation



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The authors of KEs AOPwiki ID 1392 (oxidative stress), 55 (Cell injury/death), 386 (Decrease network function), of the AO (Learning and memory, impairment), and of KER 359 (decrease network function leads to impairment in learning and memory) are greatly acknowledged.

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	WPHA/WNT Endorsed	1.13	Included in OECD Work Plan

Abstract

This Adverse Outcome Pathway (AOP) describes the linkage between binding to sulfhydryl(SH)-/seleno-proteins involved in protection against oxidative stress and impairment in learning and memory, the Adverse Outcome (AO). Binding to SH-/ seleno-proteins involved in protection against oxidative stress has been defined as the Molecular Initiating Event (MIE). Production, binding and degradation of Reactive Oxygen Radicals (ROS) are tightly regulated, and an imbalance between production and protection may cause oxidative stress, which is common to many toxicity pathways. Oxidative stress may lead to an imbalance in glutamate neurotransmission, which is involved in learning and memory. Oxidative stress may also cause cellular injury and death. During brain development and in particular during the establishment of neuronal connections and networks, such perturbations may lead to functional impairment in learning and memory. Neuroinflammation (Resident cell activation; Increased pro-inflammatory mediators) is triggered early in cell injury cascades and is considered as an exacerbating factor. The weight-of-evidence supporting the relationship between the described key events is based mainly on developmental effects observed after an exposure to the heavy metal, mercury, known for its strong affinity to many SH-/seleno-containing proteins, but in particular to those having anti-oxidant properties, such as glutathione (GSH). The overall assessment of this AOP is considered as strong, based on the biological plausibility, the empirical support and on the essentiality of the Key Events (KEs), which are moderate to strong, since blocking, preventing or attenuating an upstream KE is mitigating the downstream KE. The gap of knowledge is mainly due to limited quantitative evaluations, impeding thus the development of predictive models.

Background

This AOP was originally started in a workshop report entitled: Adverse Outcome Pathways (AOP) relevant to Neurotoxicity and published in Critical Review in Toxicol: Bal-Price, A., Crofton, K.M., Sachana, M., Shafer, T.J., Behl, M., Forsby, A., Hargreaves, A., Landesmann, B., Lein, P.J., Louisse, J., Monnet-Tschudi, F., Paini, A., Rolaki, A., Schrattenholz, A., Sunol, C., van Thriel, C., Whelan, M., Fritsche, E., 2015. Putative adverse outcome pathways relevant to neurotoxicity. Crit Rev Toxicol 45(1), 83-91.

The process of inflammation is common to many tissues and can be described by several KEs, as proposed in a dedicated workshop (Villeneuve et al., 2018). Brain inflammation called Neuroinflammation can be described by the two common KEs: Tissue resident cell, activation and pro-inflammatory mediators, increased. However, Neuroinflammation is a concept accepted by the regulators and is found in the whole literature describing brain inflammation. Therefore, in accord with the external

reviewers, we decided to use the KE Neuroinflammation for building the KERs of this AOP, but we introduced in the list of the KEs the two KEs common to the inflammatory process, as proposed in Villeneuve et al., 2018.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1487	Binding, Thiol/seleno-proteins involved in protection against oxidative stress	Binding, SH/SeH proteins involved in protection against oxidative stress
2	KE	1538	Decreased protection against oxidative stress	Protection against oxidative stress, decreased
3	KE	1392	Oxidative Stress	Oxidative Stress
4	KE	1488	Glutamate dyshomeostasis	Glutamate dyshomeostasis
5	KE	55	Cell injury/death	Cell injury/death
6	KE	188	Neuroinflammation	Neuroinflammation
7	KE	1492	Tissue resident cell activation	Tissue resident cell activation
8	KE	1493	Increased Pro-inflammatory mediators	Increased pro-inflammatory mediators
9	KE	386	Decrease of neuronal network function	Neuronal network function, Decreased
10	AO	341	Impairment, Learning and memory	Impairment, Learning and memory

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding, Thiol/seleno-proteins involved in protection against oxidative stress	adjacent	Decreased protection against oxidative stress	Moderate	Moderate
Decreased protection against oxidative stress	adjacent	Oxidative Stress	High	High
Oxidative Stress	adjacent	Glutamate dyshomeostasis	Low	Low
Glutamate dyshomeostasis	adjacent	Cell injury/death	High	Moderate
Cell injury/death	adjacent	Neuroinflammation	Moderate	
Cell injury/death	adjacent	Tissue resident cell activation	Moderate	
Neuroinflammation	adjacent	Cell injury/death	Moderate	
Increased Pro-inflammatory mediators	adjacent	Cell injury/death	Moderate	
Cell injury/death	adjacent	Decrease of neuronal network function	Moderate	
Decrease of neuronal network function	adjacent	Impairment, Learning and memory	High	
Oxidative Stress	non-adjacent	Cell injury/death	High	High

Stressors

Name	Evidence
Methylmercuric(II) chloride	High
Mercuric chloride	High
Acrylamide	Low

Overall Assessment of the AOP

Experimental and epidemiological evidences indicate that compared to the adult central nervous system (CNS), the developing CNS is generally more susceptible to toxicant exposure (Costa et al., 2004; Grandjean and Landrigan, 2006). Pre-natal and post-natal exposure may have long-term consequences, i.e. not detected immediately at the end of the exposure period. Such effects on visuospatial memory for example have been described on child development in communities with chronic low level mercury exposure (Castoldi et al., 2008a; Debes et al., 2006; Grandjean et al., 2014; Lam et al., 2013).

The aim of this AOP is to capture the KEs and the KERs that occur after binding to thiol- and selenol groups of proteins involved in protection against oxidative stress, the MIE, and impairment in learning and memory, the AO, which is a neurotoxicity marker belonging to the OECD regulatory tool box. The chemical initiators used for the empirical support are methylmercury and mercury chloride, and acrylamide. Data are most extensive for mercury as stressor during development; data for acrylamide are much more limited and restricted to some KEs. Chronic, low-dose prenatal MeHg exposure from maternal consumption of fish has been associated with endpoints of neurotoxicity in children, including poor performance on neurobehavioral tests, particularly on tests of attention, fine-motor function, language, visual-spatial abilities (e.g., drawing), and verbal memory (NRC, 2000). However, it is important to note that some uncertainties remain about the effects of low dose of mercury during brain development (Grandjean et al., 1999). Epidemiological studies in Seychelles on prenatal exposure through fish consumption did not evidenced adverse effects on memory when analyses were performed at 22 and 24 years (Van Wyngaarden et al., 2017), whereas similar experiments made in the Faroe Islands revealed dysfunctions in language, attention and memory at 7 years (Grandjean et al., 1997). And a clear association was observed between mercury cord blood level and memory deficit (Grandjean et al., 1997; Debes et al., 2006). Castoldi and coworkers (2008) proposed that modulating factors, such as diet, nutrition, gender, pattern of exposure and co-exposure could explain the discrepancies of these epidemiological studies. Nevertheless, there are experimental evidences showing that the neurocognitive domain, in particular dentate gyrus, hippocampus and cortex are susceptible to the neurotoxicity of mercury in the developing brain (Sokolowski et al., 2011, 2013; Ceccatelli et al., 2013); therefore, we focus on impairment in learning and memory as the AO. Some –SH- or –SeH-containing proteins involved in protection against oxidative stress have been demonstrated to be inhibited by MeHg either *in vitro* or *in vivo*, but a causal relationship has not been established between these inhibitory effects and the final

pathological events (Oliveira, 2017). However, the analysis of the essentiality of the KEs and of the weight of evidence for the KERs supports a plausible mechanistic link between the MIE and the AO.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
During brain development	

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Male	
Female	

This AOP is mainly focused on the developmental period, although it cannot be excluded that long-term exposure in adult may trigger a similar cascade of KEs leading also to impairment in learning and memory, as observed in neurodegenerative diseases such as Alzheimer's disease (Mutter et al., 2004). While no specific sex differences have been analyzed/described for most KEs, Curtis and coworkers (2010) observed a higher level of TNF-a in hippocampus of male prairie wolf than in female, both treated for 10 weeks with inorganic mercury, in the form of $HgCl_2$; whereas Zhang and coworkers (2013) found a higher neuroinflammatory response associated with altered social behavior in female mice offspring than in male, following gestational exposure to $HgCl_2$. However, after developmental methylmercury exposure, long-lasting behavioral alterations were more prominent in males (Ceccatelli et al., 2013; Castoldi et al., 2008b). These discrepancies may be due to sex differences in kinetics or susceptibility (Vahter et al., 2006).

Essentiality of the Key Events

KE	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 (e.g. stop/reversibility studies, antagonism, KO models, etc.)	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE leading to increase in KE down or AO	No or contradictory experimental evidence on the essentiality of any of the KEs
KE1 **Decreased protection against oxidative stress**	HIGH	RATIONALE: The fact that a decrease in anti-oxidant properties causes oxidative stress is well accepted. In addition, experimental evidences of knocking out proteins involved in protection against oxidative stress incresed the susceptibilty to oxidative stress.		
KE2 **Oxidative stress**	HIGH	RATIONALE: The deleterious consequences of oxidative stress are well accepted in various animal models. Oxygen radical scavengers, such as glutathione, catalase, selenium and cysteine can block the deleterious effects of oxidative stress.		
KE3 **Glutamate dyshomeostasis**	HIGH	RATIONALE: Glutamate is the main excitatory transmitter, and is involved in memory processes, it is well accepted that perturbation of glutamate homeostasis has deleterious functional consequences. Disruption of glutamate signaling is thought to play a role, at least in part, in the etiology underlying several neurodevelopmental disorders, including memory dysfunction.		
KE4 **Cell Injury/death, increased**	HIGH	RATIONALE: Cell injury/death is a highly converging node in AOPs. Decrease in synaptic connectivity or cell loss will in turn induce perturbations in the establishment of neuronal connections and trigger inflammatory responses, which through a feedback loop can exacerbate this KE. Therefore, prevention of cell injury/death by anti-oxidant or by inhibitors of NMDA receptors prevents the downstream KEs.		
KE5 **Neuroinflammation** **KE5' Tissue resident cell activation** **KE5'' Pro-inflammatory mediators, increased**	MODERATE	RATIONALE: It is widely accepted in different experimental animal models that the use of minocycline, an antibiotic, which blocks microglial reactivity has protective effects, as have other interferences with any inflammatory mediators. However, we rate the essentiality of this KE as moderate given the complexity of the neuroinflammatory response, having either protective/reparative or aggravating consequences,		
KE6 **Decreased network formation and function**	HIGH	RATIONALE: Glutamate neurotransmission is an important mechanism underlying memory function (for review: Featherstone, 2010). During brain development, glutamate has also trophic effects, by stimulating BDNF production or through the activation of the different glutamate receptors. The trophic effect of glutamate receptor activation is developmental stage-dependent and may play an important role in determining the selective survival of neurons that made proper connections (Balazs, 2006).		
AO **Impairment of learning and memory**	HIGH	RATIONALE: Impairment in learning and memory is a converging KE in several AOPs related to brain development. Regarding this AOP and its chemical initiators, it was shown that the neurocognitive domain, in particular dentate gyrus, hippocampus and cortex are susceptible to the neurotoxicity of mercury in the developing brain (Sokolowski et al., 2011, 2013; Ceccatelli et al., 2013). Chronic, low-dose prenatal MeHg exposure from maternal consumption of fish has been associated with endpoints of neurotoxicity in children, including poor performance on neurobehavioral tests, particularly on tests of attention, fine-motor function, language, visual-spatial abilities		

Weight of Evidence Summary

Dose-response and temporal concordance of KEs

There is no study where all KEs are measured simultaneously after exposure to several doses, impeding a dose-response and concordance analysis. In one single study (in blue in the table), three downstream KEs were measured following pre-natal exposure to methylmercury. Comparisons of all animal studies show that doses used are ranging from 0.5 - 5 mg/kg; but dose-response was seldom performed. In these studies, the time (pre-natal, post-natal, lactation,...) and duration of exposure are quite diverse and no analysis of brain mercury content was made, so it is not possible to compare doses between studies. Therefore, based on the present data, it is impossible to define whether KEs up occur at lower doses and earlier time points than KEs down.

For *in vitro* studies, KEs up are often measured after acute exposure to high concentrations.

The following table summarizes concentrations/doses, time, and duration of exposure for the various test systems and KEs.

Biological Plausibility and Empirical Support of the KERs

		(e.g., drawing), and verbal memory (NRC, 2000). Prenatal MeHg exposure is associated with childhood memory and learning deficits, particularly visual memory performance in school-aged children (Orenstein, 2014).

KEs	In vivo	In vitro
MIE **Binding to SH-/seleno-proteins**		Binding of Hg to thiol groups and to various selenium-containing proteins: Glutathione, thioredoxin reductase, thioredoxin, glutaredoxin, glutathione reductase was measured using purified proteins (Carvahlo et al., 2008, 2011; Wiederhold et al., 2010; Sugiura et al., 1978; Arnold et al., 1986; Han et al., 2001; Qiao et al., 2017)
KE1 **Decreased protection against oxidative stress**	Cytoplasmic and nuclear TrxR and Cytoplasmic Gpx were reduced in cerebral and cerebellar cortex of 22 days-old offspring (Ruszkiewicz, 2016) Male C57BL/6NJcl mice exposed to methylmercury (1.5 mg/kg/day for 6-weeks) (Fujimura, 2017) Adult male Sprague-Dawley rats exposed to methylmercury (1 mg/kg orally for 6 months) (Joshi, 2014) Zebra fish brain exposed to Hg2+, MeHg 1.8 molar (measured in brain tissue), for 28 days (Branco, 2012) Prenatal and postnatal exposure of mice to 40 ppm of $HgCl_2$ decreased the activity of catalase, thioredoxin reductase, Gpx, superoxide dismutase (Malqui et al., 2017)	Mouse primary cortical cultures exposed to 5 mM of methylmercury for 24h (Rush, 2012) MeHg inhibits ex vivo rat thioredoxin reductase; IC50 0.158 µM (cerebral) (Wagner et al., 2010) Human neuroblastoma cells (SH-SY5Y)exposed to 1 µM of methylmercury for 6 or 24 h (Branco, 2017; Franco, 2009)
KE2 **Oxidative stress**	Male C57BL/6NJcl mice exposed to methylmercury (1.5 mg/kg/day for 6-weeks) (Fujimura, 2017) Adult male Sprague-Dawley rats exposed to methylmercury (1 mg/kg orally for 6 months) (Joshi, 2014) Adult male Sprague-Dawley rats exposed to methylmercury (1 mg/kg orally for 6 months) (Joshi, 2014) Zebra fish brain exposed to Hg2+, MeHg 1.8 molar (measured in brain tissue), for 28 days (Branco, 2012) Prenatal and postnatal exposure of mice to 40 ppm of $HgCl_2$ caused oxidative stress evaluated by increased lipid peroxidation (Malqui et al., 2017)	Mouse primary cortical cultures exposed to 5 mM of methylmercury for 24h (Rush, 2012) Methylmercury (2-10 µM) in synaptic vesicles isolated from rat brain (with LD_{50} at 50 µM) (Porciuncula et al., 2003) Human neuroblastoma cells (SH-SY5Y)exposed to 1 µM of methylmercury for 6-24 h (Franco, 2009)
KE3 **Glutamate dyshomeostasis**	Rat Young (3-4 weeks) dosed with acrylamide by gavage (5, 15, 30 mg/kg, 5 applications per week during 4 weeks) (Tian, 2018) Microdialysis probe in adult Wistar rats showed that acute exposure to methylmercury (10, 100 mM) induced an increase release of extracellular glutamate (9.8 fold at 10 mM and 2.4 fold at 100 mM). This extracellular glutamate level remained elevated at least 90 min (Juarez et al., 2002)	Mouse astrocytes, neurons in mono- or co-cultures exposed to methylmercury 1-50 µM for 24h (Morken, 2005) Methylmercury (2-10 µM) in synaptic vesicles isolated from rat brain (with LD_{50} at 50 µM) (Porciuncula et al., 2003
KE4 **Cell Injury/death, increased**	Rat, perinatal exposure to methylmercury (GD7-PD21, i.e. 35 days) 0.5 mg/kg bw/day in drinking water (Roda et al., 2008) Rat Young (3-4 weeks) exposed to acrylamide by gavage (5, 15, 30 mg/kg, 5 applications per week during 4 weeks) (Tian, 2018) Pregnant rat exposed to methylmercury (1.5 mg/kg orally) from GD5 till parturition (Jacob, 2017)	Mouse astrocytes, neurons in mono- or co-cultures exposed to methylmercury 1-50 µM for 24h (Morken, 2005)
KE5 **Neuroinflammation** **KE5' Tissue resident cell activation** **KE5'' Pro-inflammatory mediators, increased**	Rat, perinatal exposure to methylmercury (GD7-PD21, i.e. 35 days) 0.5 mg/kg bw/day in drinking water (Roda et al., 2008) Monkeys, 6,12,18 months oral exposure 50 mg/kg bw (Charleston et al., 1996)	3D rat brain cell cultures 10 day treatment$HgCl_2$ 10^{-9}-10^{-6}M MeHgCl 10^{-9}-$3x10^{-7}$M (Monnet-Tschudi et al., 1996; Eskes et al., 2002)
KE6 **Decreased network formation and function**	Mice dosed during postnatal week 1-3 with subcutaneous 2-5 mg mercury chloride/kg/once per week (Eddins et al., 2008) Pregnant rat dosed on GD 15 with 8 mg/kg of methylmercury by gavage. Offsprings were tested at day 16, 21	

	and 60. (Cagiano et al., 1990) Pregnant rat exposed to methylmercury (1.5 mg/kg orally) from GD5 till parturition (Jacob, 2017)	
AO **Impairment of learning and memory**	Mice dosed during postnatal week 1-3 with subcutaneous 2-5 mg mercury chloride/kg/once per week (Eddins et al., 2008) Pregnant rat dosed on GD 15 with 8 mg/kg of methylmercury by gavage. Offsprings were tested at day 16, 21 and 60 (Cagiano et al., 1990) Pregnant rat exposed to methylmercury (1.5 mg/kg orally) from GD5 till parturition (Jacob, 2017) Pregnant mice received 0.5 mg methylmercury/kg/day in drinking water from gestational day 7 until day 7 after delivery. Offspring behavior was monitored at 5-15 and 26-36 weeks of age (Onishchenko et al., 2007) 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) (Glover et al., 2009) Prenatal and postnatal exposure of mice to 40 ppm of $HgCl_2$ caused impairment of memory (object recognition, Y maze) Malqui et al., 2017)	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 µg/g, similar to fish-eating population in US. Statistical analyses revealed that each ug/g increase in hair Hg was associated with a decrement in visual memory, learning and verbal memory (Orenstein et al., 2014) Epidemiological studies in the Faroe Islands revealed that mercury exposure through fish consumption (maternal hair conc. 10 ug/g) dysfunctions in memory, language and attention at age 7 (Grandjean et al., 1997; Debes et al., 2006)

KERs	Defining Question Is there a mechanistic (i.e. structural or functional) relationship between KEup and KEdown consistent with established biological knowledge?	High (Strong) Extensive understanding of the KER based on extensive previous documentation and broad acceptance	Moderate The KER is plausible based on analogy to accept biological relationship but scientific understanding is not completely established	Low (Weak) There is empirical support for a statistical association between KEs but the structural or functional relationship between them is not understood
MIE to KE Decrease protection against oxidative stress	MODERATE	RATIONALE: Thiol- and selenol containing proteins, which mainly belong to the anti-oxidant protections, have a high affinity for binding soft metals such as mercury (Farina, 2011). Binding to these thiol/sulfhydryl/SH/SeH groups results in structural modifications affecting the catalytic capacity, and thereby reducing the capacity to neutralize ROS. However, binding to other SH/SeH groups of other proteins not involved in protection against oxidative stress can occur and trigger other neurotoxicity pathways. Alternatively, binding to SH groups of electrophilic compounds may also induce cyto-protective reactions (e.g. via Nrf2).		
KE Decrease protection against oxidative stress to KE Oxidative stress	HIGH	RATIONALE: Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses. Several studies have shown depletion of GSH, the main anti-oxidant, and an increase in oxidative stress following methylmercury or mercury chloride exposures (Meinerz, 2011; Rush, 2012; Agrawal, 2015). Protection against oxidative stress was observed by supplementation with diphenyl selenide (Meinerz, 2011) or by glutathione ester (Rush, 2012). Limited conflicting data.		
KE Oxidative stress to KE Glutamate (Glu) dyshomeostasis	LOW	RATIONALE: Glutamate transport is driven by the Na^+ ion gradient, which is dependent on the Na/K ATPase, which, in turn, requires energy. Glutamate enters the cells accompanied by 2 Na^+ and one H^+. Perturbations of energy metabolism such as mitochondrial dysfunction and increased production of ROS will lead to glutamate dyshomeostasis, due to the indirect coupling of glutamate transporters with ATP level, and to the important role of glutamate transporters in glutamate homeostasis. (Boron and Boulpaep, 2003). Methylmercury was shown to inhibit both the H^+-ATPase activity and vesicular glutamate uptake (Porciuncula et al., 2003). As, on one hand, ROS production can interfere with glutamate uptake, and on the other hand, glutamate accumulation leads to excitotoxicity and ROS production, the exact sequence of the KER is difficult to assess. But the fact that both KEs are involved in mercury-induced neurotoxicity is broadly accepted (Farina et al., 2011; Antunes dos Santos et al., 2016; Morris et al., 2017; Kern et al., 2016).		
KE Glutamate dyshomeostasis to KE Cell injury/death	HIGH	RATIONALE: Glutamate dyshomeostasis, in particular excess of glutamate in the synaptic cleft, leads to overactivation of ionotropic glutamate receptors, referred to as excitotoxicity. This, in turn, will cause cell injury/death via ROS production. This KER is also inherent to the developing brain, where glutamate ionotropic receptors are expressed early in various neural cells and when NMDA receptors are expressed in neurons. There is empirical support for all three chemical initiators (mercury, acrylamide, acrolein). In addition, several experiments aiming at blocking glutamate excitotoxicity and the resulting ROS production are protective for cell injury/death. Limited conflicting data.		
KE Cell injury/death to KE Neuroinflammation	MODERATE	RATIONALE: It is widely accepted that cell/neuronal injury and death lead to neuroinflammation (microglial and astrocyte reactivities) in adult brain, and in the developing brain, where neuroinflammation was observed after cell injury/death induced by excitotoxic lesions (Acarin et al., 1997; Dommergues et al., 2003). Empirical support is available for all three chemical initiators (mercury, acrylamide, acrolein). Few experiments, showing a protection when blocking any feature of neuroinflammation have been described. There are some contradicting data showing an absence of neuroinflammatory response despite the occurrence of mercury-induced apotosis and slight behavioral alterations.		
KE Neuroinflammation to KE Cell injury/death	MODERATE	RATIONALE: In vitro co-culture experiments have demonstrated that reactive glial cells (microglia and astrocytes) can kill neurons via the release of pro-inflammatory cytokines, such as TNF-a, IL-1b and IL-6 and/or ROS/RNS (Chao et al., 1995; Brown and Bal-Price, 2003; Kraft and Harry, 2011; Taetzsch and Block, 2013) and that interventions aiming at blocking these inflammatory biomolecules can rescue the neurons (Yadav et al., 2012; Brzozowski et al., 2015). Several reports showed that modulating mercury or acrylamide-induced neuroinflammation was protective for neurons. Because of the complexity of the neuroinflammatory response, that can have neuroprotective or neurodegenerative consequences depending on the duration, local environment or still unknown factors, the rating of this KER was kept as moderate. The vicious cycle between cell injury/death and neuroinflammation is well known and was described in other AOPs. Neuroinflammation could be considered as a modulating factor, but because of the numerous inhibiting experiments, it is considered as an essential KE. Some conflicting data due to the dual role of some inflammatory mediators have been reported.		
KE Cell injury/death to	HIGH	RATIONALE: Neuronal network formation and functional crosstalk are established via		

KE Decreased network formation and function		synaptogenesis. It was shown that under physiological conditions components of the apoptotic machinery in the developing brain regulate synapse formation and neuronal connectivity (Dekkers et al., 2013). The brain's electrical activity dependence on synapse formation is critical for proper neuronal communication. Glial cells are also involved in the establishment and stabilization of the neuronal network. Extensive experimental support for the adverse effects of mercury on synaptogenesis exist, establishing a strong link between mercury-induced apoptosis and/or neuronal loss and perturbations in a number of neurotransmitter systems (Jacob, 2017; Bridges, 2017) and perturbations of functionality (Falluel-Morel, 2007; Ferraro, 2009; Teixera, 2014; Onishchenko, 2007). Limited protective experiments and conflicting data reported.
KE Decreased network formation and function to AO Impairment in learning and memory	HIGH	RATIONALE: A review on the Morris water maze (MWM) (Morris, 1981), as an investigative tool of spatial learning and memory in laboratory rats (Vorhees and Williams, 2006) pointed out that perturbed neuronal networks rather than neuronal death per se in certain regions is responsible for the impairment in MWM performance. Functional integrated neural networks that involve the coordination action of different brain regions are consequently important for spatial learning and memory performance (D'Hooge and De Deyn, 2001). Broad empirical support showing mercury-induced effects on learning and memory as consequence of network disruption (Sokolowski et al. 2013; Eddins et al., 2008; Glover et al., 2009). Similar observations were made in humans (Orenstein et al., 2014; Yorifuji et al., 2011). Interestingly, behavioral alterations were detected long time after exposure (delayed effects). Few conflicting data have been reported, but other behavioral deficits, such as alterations in motor activity and increased anxiety suggest that systems other than hippocampus-related learning and memory are also affected.
KE oxidative stress to KE Cell injury/death	HIGH	RATIONALE: The central nervous system is especially vulnerable to free radical damage since it has a high oxygen consumption rate, an abundant lipid content and reduced levels of antioxidant enzymes (Coyle and Puttfarcken, 1993; Markesbery, 1997). The developing nervous system is particularly vulnerable to chemical insults (Grandjean and Landrigan, 2014). One reason for this higher vulnerability is the incapacity of immature neural cells to cope with oxidative stress by increasing glutathione (GSH) production (Sandström et al., 2017a). Broad empirical support for mercury and acrylamide showing an association between increased ROS production and/or decreased protection against oxidative stress and apoptosis and/or necrosis (Lu *et al.*, 2011; Sarafian *et al.*, 1994; Allam *et al.*, 2011; Lakshmi *et al.*, 2012). Anti-oxidant treatments proved to be protective. Few conflicting data, except a mercury-induced upregulation of GSH level and GR activity as an adaptive mechanism following lactational exposure to methylmercury (10 mg/L in drinking water) associated with motor deficit, suggesting neuronal impairment (Franco et al., 2006).

Quantitative Consideration

Some quantitative relationships have been described between the upstream early KEs (MIE, oxidative stress, Cell injury/death), although the diversity of test systems and posology (dosing/exposure amount and duration) hampers comparison between studies. It is more difficult to evaluate quantitative relationships between later downstream KEs, such as Neuroinflammation and Decreased Network Function. Neuroinflammation is a complex adaptive mechanism which is not yet completely understood; it can have neuroprotective or neurodegenerative consequences, depending on triggering signals, duration, microenvironment or other unknown influences, which may determine the outcome of the neuroinflammatory process. Decreased network function is currently difficult to quantify because quantitative technologies for mapping and understanding of brain networks (and their plasticity) are still under development.

Optimally, we would like data from a single type of test system showing that exposure to stressor, e.g. mercury, is correlated with changes in all KEs. Such models are emerging, using cells of human origin (Pamies et al., 2016; Sandström et al., 2017b; Fritsche et al., 2017) and/or non-mammalian models, such as zebrafish (Geier et al., 2018; Padilla et al., 2018) and will allow in the future generation of quantitative data which may be used for *in silico* hazard prediction.

Summary table of Quantitative Evaluations

KEs	Methylmercury (MeHg, CH_3Hg)			
	5 µM mouse brain *in vitro* (Rush, 2012)	15–30 µM mouse brain, after 40 mg/L in drinking water for 21 days (Glaser, 2013)	1 µM mouse cerebral cortex ex vivo after oral dosing (Lu et al 2011; conc. from Huang et al 2008)	17-24, 75-µM (rat cerebral cortex *ex vivo* after 4w ip dosing) 4w (Xu, 2012; Liu 2013; Feng, 2014)
KE1 **Decreased protection against oxidative stress**	GSH reduced 80% of control 24h	Cortical mitochondrial GPx activity decreased (70% of control), GR increased (170% of control)	GSH decreased (ca 50% of control) 7 weeks	Antioxidants NPSH, SOD, GSH-Px decreased (ca 80% and 50% of control)
KE2 **Oxidative stress**	ROS increased 120-150% of control 24h	Cortical mitochondrial TBA-RS increased (ca 140% of control) and complex I, II-III, and IV activity decreased (ca 50% of control). Brain 8-OHdG content increased (ca 400% of control).	LPO increased (ca 200% of control) 7 weeks	ROS (DCF) increased (190 and 400% of control at 22,87 µM)
KE3 **Glutamate dyshomeostasis**				Glutamine synthetase decreased (80 and 50% of control at 24,89 µM) Glutamate content increased (100 and 120% of control at 24,89 µM) Glutamine content decreased (80 and 50% of control at 24,89 µM)
KE4 **Cell Injury/death, increased**			Apoptosis-related gene expression: Bcl-2 decreased, ca 50% of control; Bax, Bak, p53, caspase-3,-5,-7 increased, ca 200-350% of control 7 weeks	Apoptosis increased dose-dependently (300 and 853% of control at 24,89 µM). 8-OHdG expression increased (200 and 450% of control at 24,89 µM)
KE5 **Neuroinflammation**				
KE6 **Decreased network formation and function**				

AO **Impairment of learning and memory**				
KEs	Mercuric chloride ($HgCl_2$)			
	6 µM rat brain, 1.13 µg Hg/g 6 mo (Agrawal, 2015)	0.1-100 µM cultured mouse cerebellar granule cells 10 min (Fonfria, 2005)		
KE1 **Decreased protection against oxidative stress**	Blood GSH decreased (ca 90% of control)			
KE2 **Oxidative stress**				
KE3 **Glutamate dyshomeostasis**		Glutamate (3H-aspartate) uptake inhibited (IC50 3.5 uM). Glutamate release stimulated (47% of total endogenous glutamate at 10 µM)		
KE4 **Cell Injury/death, increased**	Serum AST increased (ca 140% of control).	Cell viability (MTT) decreased (ca 10% of control at 10 µM)		
KE5 **Neuroinflammation**				
KE6 **Decreased network formation and function**	Brain noradrenaline and dopamine content decreased (ca 30% of control).			
AO **Impairment of learning and memory**				

Considerations for Potential Applications of the AOP (optional)

- Contribution to the network of KEs/AOPs on Developmental Neurotoxicity (DNT)
- Generating quantitative data by measuring all KEs in a single model after repeated/long term exposure to a wide concentration range of the chemical initiators to facilitate the development of computational predictive approaches

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Appendix 1

List of MIEs in this AOP

Event: 1487: Binding, Thiol/seleno-proteins involved in protection against oxidative stress

Short Name: Binding, SH/SeH proteins involved in protection against oxidative stress

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	MolecularInitiatingEvent
Aop:284 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress leads to chronic kidney disease	MolecularInitiatingEvent

Stressors

Name
Methylmercuric(II) chloride
Mercury chloride
Acrylamide

Biological Context

Level of Biological Organization
Molecular

Organ term

Organ term
brain

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Mercury (Methylmercury, mercury chloride)

The binding of Methylmercury (MeHg) to redox sensitive thiol- or selenol-groups can disrupt the activity of enzymes or the biochemical role of non-enzymatic proteins. The stable or transitory interaction (binding) of MeHg with critical thiol and selenol groups in target enzymes can disrupt the biological function of different types of enzymes, particularly of the antioxidant selenoenzymes thioredoxin reductase (TrxR) and glutathione peroxidase isoforms. The dysregulation of cerebral glutathione (GSH and GSSG) and thioredoxin [Trx or $Trx(SH)_2$] systems by MeHg (Farina et al. 2011; Branco et al. 2017) can impair the fine cellular redox balance via disruption of sensitive cysteinyl- or thiol-containing proteins (Go etal., 2013; Go et al. 2014; Jones 2015).

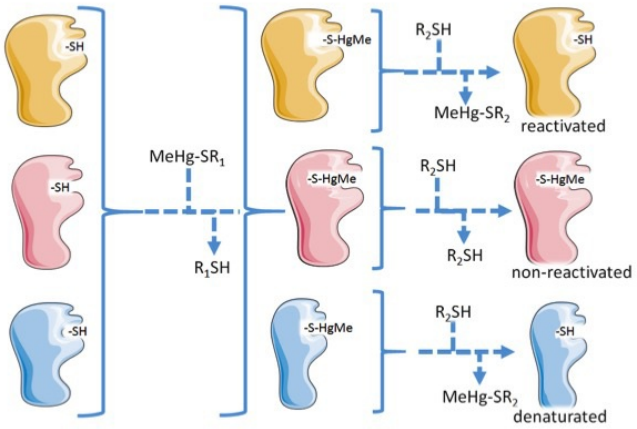

Figure 1 – Hypothetical Binding of MeHg to different types of target proteins. The binding of MeHg to proteins can cause either a transitory inhibition of the protein fucntion (first line, the yellow protein was reactivated by interacting with LMM-SH or R-SH). The pink protein is an example of protein that after the binding of MeHg suffered a change in the structure in such a way that it cannot be reactivated by LMM-SH or R-SH. The third protein (blue) is an example of protein that was permanently denaturated after MeHg binding and even after the removal of MeHg the activity was not recovered. The same type of interactions can be applied to the selenol-containing proteins (i.e., the selenoproteins).

The affinity of Mercury chloride (Hg^{2+}) for thiol and selenol groups is higher than that of MeHg (compare Table 2 with Table 1). The constants described in Table 1 and 2 indicate that MeHg and Hg^{2+} behave as strong soft electrophiles, i.e., theyhave much higher affinity for the soft nucleophiles centers of thiol- and selenol-containing molecules (Rabenstein 1978a; Arnold et al. 1986; Sugiura et al., 1976).Furthermore, the rate constant for the reaction of MeHg with thiol/thiolate (R-SH/R-S^-) has been estimated to be about 6 x 10^8 $M^{-1}.sec^{-1}$, indicating that the reactions of electrophilic forms of Hg ($EpHg^+$; here MeHg and Hg^{2+}) with thiolate and selenolate groups are diffusion controlled reactions (Rabenstein and Fairhurst, 1975). The constant indicates that the binding of $EpHg^+$ to thiolate (-S^-) or selenolate (-Se^-) groups will occurr almost instaneously, when an $EpHg^+$ collides with –S^- or -Se^- groups.

The studies of Rabenstein and others have also pointed out that the affinity of MeHg for –SeH groups is higher than for –SH groups (Sugira et al. 1976; Arnold et al. 1986). Consequently, –SeH-containing molecules (i.e., selenoproteins) should be the preferential targets for MeHg (Farina et al. 2011). Accordingly, several studies have demonstrate that the selenoenzymes glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) were inhibited after *in vitro* and *in vivo* exposure to MeHg or Hg^{2+} (Carvalho et al., 2008a; 2011, Farina et al., 2009; Franco et al., 2009; Wagner et al., 2010; Branco et al., 2011; 2012; 2014, 2017; Dalla Corte et al., 2013; Meinerz et al., 2017).

As corollary, the occurrence of free MeHg and Hg^{2+} or bound to other ligands such as carboxylates, amines, chloride or hydroxyl anions in the physiological media of living cells is insignificant or nonexistent (George et al. 2008). The binding of MeHg to abundant low molecular mass thiols or LMM-SH (e.g., cysteine and reduced glutathione-GSH) and high molecular mass thiol-containing proteins or HMM-SH (e.g., albumin, hemoglobin, etc) is critical for the MeHg distribution from non-target to target organs and cells (Farina et al. 2017). The coordination of MeHg with one –S^- group of a LMM-SH will determine MeHg distribution to its targets organs, including the brain. The coordination of Hg^{2+} with two –S^- of LMM-SH molecules (particularly, cysteine or Cys) will determine the distribution of Hg^{2+} to kidney (which is its main target) and to non-classical targets organs, such as the brain (Oliveira et al. 2017). The entrance of Hg^{2+} into the brain is proportionally small, but recent literature data have indicated the neurotoxicity of very low and environmentally relevant doses of Hg^{2+} in rodents (Mello-Carpes et al. 2013), which confirms data obtained with toxic doses in rodents (Peixoto et al. 2007 ; Franciscato et al. 2009 ; Chehimi et al. 2012).

Table 1 - Affinity constants of methylmercury for important chemical groups found in biomolecules (adapted from [a]Rabestein, 1978a, [b]Rabestein and Bravo, 1987, using different thiol-containing molecules with the arylmercurialpara-mercurybenzenosulfonate, and from [c]Arnold et al. 1986 taking into consideration that the calculated formation constant of –Se-MeHg conjugates was 0.1 to 1.2 order greater than that of –S-MeHg). The values represent the Log of the constants.

Functional Group	Occurrence	Formation constant
Thiol/thiolate (-SH/-S^-)	Cysteine, glutathione, proteins	≈14-18 [a,b]
Selenol/selenolate (-SeH/Se^-)	Selenocysteinyl residues in selenoproteins	≈ 16-18[c]

Table 2. Formation constants of Hg^{2+} with some representative nucleophilic centers from biomolecules.

Functional group	Hg^{2+}
R-S-R	≈ 6-12
R-SH	≈ 40-50
R-SeH	≈ 50-60

The approximate (≈) Log of the constants. The values were adapted from Stricks and Kolthoff 1953; Mousavi 2011 and Liem-Nguyem et al. 2017.

We have to emphasize that what we call of binding to –SH or –SeH groups is, in fact, an exchange reaction of MeHg from MeHg-S conjugates (e.g., MeHg-cysteine or MeHg-Cys and MeHg-glutathione or MeHg-SG. conjugates) to a free thiol/thiolate- or selenol/selenolate-group from non-target or target proteins. Thus, the interaction of MeHg with its target proteins in the brain usually involves the exchange of MeHg from low-molecular mass conjugates (LMM-S-conjugates) to a thiol or selenol group in different types of proteins (Rabenstein 1978b; Rabenstein and Fairhurst, 1975; Reid and Rabenstein et al.; 1982; Rabenstein and Reid, 1984; Arnold et al. 1986; Farina et al. 2011, 2017; Dórea et al. 2013).

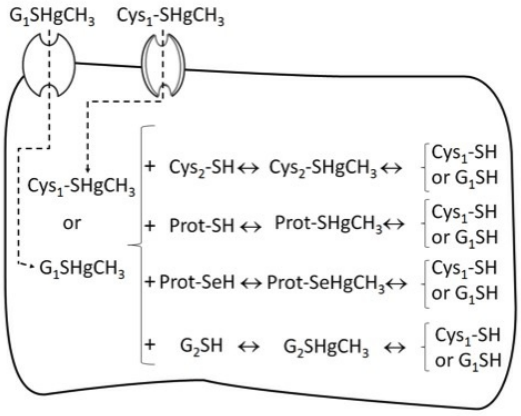

Figure 2 – Binding of MeHg (CH3Hg+) to target thiol- (HMM-SH) or selenol-containing proteins (HMM-SeH). Note that, in fact, the binding of MeHg to their high molecular mass target proteins is mediated by exchange reactions of MeHg from low molecular mass thiol (LMM-SH) molecules to HMM-SH (represented by Prot-SH) or HMM-SeH (represented by Prot-SeH). The scheme also demonstrated that MeHg conjugated with one LMM-SH (here represented by either Cys_1-$SHgCH_3$ or G_1SHgCH_3) can exchange with others LMM-SH (here represented by Cys_2-SH or G_2SH). After one exchange reaction, the conjugated Cys_1-$SHgCH_3$ and G_1SHgCH_3 release the free LMM-SH molecules Cys_1-SH or G_1SH.

Table 3: References for the inhibition by MeHg and Hg^{2+} of SH-/seleno-proteins involved in protection against oxidative stress

Protein activity inhibited by MeHg	**Exposure**	**Functional group likely involved in the inhibition**	**Organism-preparation**	
Glutathione peroxidase (total GPx)	*in vivo*	-SeH	Adult mice	Glasser et al. 2013
Total GPx	*in vivo*	-SeH	Adult mice	Glasser et al. 2010a
Mitochondrial total GPx	*in vivo*	-SeH	Adult mice	Franco et al. 2009
Total GPx	*in vitro*	-SeH	SH-SY5Y cells	Franco et al. 2009
GPx1 and GPx4	*in vivo*	-SeH	Adult mice	Zemolin et al. 2012
Total GPx	*in vivo*	-SeH	Adult male mice	Malagutti et al. 2009
Total GPx	*in vitro*	-SeH	PC12 cells	Li et al. 2008
Total GPx	*in vivo*	-SeH	Mice gestational exposure	Stringari et al. 2008
Total GPx	*in vivo*	-SeH	Adult rats	Cheng et al. 2005
Total GPx	*in vitro*	-SeH	Fetal Telencepalic cells from rats	Sorg et al. 1998
Total GPx	*in vitro*	-SeH	Mice neuroblastoma cells	Kromidas et al. 1990
Thioredoxin Reductase (TrxR)	*in vivo*	-SeH and –SH	Adult mice	Zemolin et al. 2012
TrxR	*in vitro*	-SeH and –SH	Adult mice	Wagner et al. 2010
TrxR	*in vivo*	-SeH- and –SH	Adult rats	Dalla Corte et al. 2013
Mitochondrial total Gpx	*In vivo*	-SeH	Adult rat	Mori et al., 2007
Mitochondrial total Gpx	*In vivo*	-SeH	Adult Swiss male mice brain	Franco et al., 2009

Total brain TrxR	*In vivo*	-SeH and -SH	Juvenile fish (zebra-seabreams)	Branco et al. 2011 Branco et al. 2012a,b
Protein activity inhibited by Hg2+	**exposure**	**Functional group likely involved in the inhibition**	**organism-preparation**	
Total brain TrxR	*In vivo*	-SeH and -SH	Juvenile fish (zebra-seabreams)	Branco et al. 2012a,b

Acrylamide

Acrylamide is an a,β-unsaturated (conjugated) reactive molecule, which can react with thiol (-SH) and amino (-NH2) groups in proteins (LoPachin, 2004; LoPachin et al. 2007; 2009; 2011; Friedman, 2003; Bent et al. 2016; Martyniuk et al.2011; LoPachin and Gavin, 2014). However, the rate constant for the reaction between acrylamide with thiol/thiolate groups is much lower than that for MeHg. The rate of reaction of this compound with HMM-SH and LMM-SH is slow but can occur under physiological conditions (Tong et al. 2004; LoPachin, 2004). The inhibition of brain enzymes by acrylamide have been studied and the inhibition caused by acrylamide in some HMM-SH can be reversible (Howland et al. 1980). Despite of this, we can infer that some targets of MeHg and acrylamide can overlap, in particular GSH,where the rate constant for MeHg and acrylamide are ≈6.0 x 10^8 $M^{-1}.sec^{-1}$ and ≈0.15-2.1 x 10^{-2} $M^{-1}.sec^{-1}$, respectively (Yousef and Demerdash, 2006; Lapadula et al. 1989; Kopańska et al. 2015). Acrylamide can also be metabolized to an epoxide intermediate (glycidamide), which can also form adducts with cysteinyl residues in HMM-SH target proteins (Bergmark et al. 1991).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus		NCBI
mouse	Mus musculus		NCBI
zebra fish	Danio rerio		NCBI
human	Homo sapiens		NCBI
Gallus gallus	Gallus gallus		NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

Due to the ubiquitous distribution of the SH-/ and seleno-proteins involved in protection against oxidative stress and inview of the strong affinity of MeHg and Hg^{2+} for thiolate and selenolate groups the binding of MeHg and Hg^{2+} to thiol and selenol groups is expected to occur in the living cells of all taxonomic groups found in the biosphere.The conservation of these effects across different vertebrate species indicates that thiol- and selenol-containing proteins (particularly, TrxR and GPx) can also be important targets of electrophilic forms of Hg($EpHg^+$ or MeHg and Hg^{2+}) toxicity in fish and birds (Heinz, 1979; Carvalho et al. 2008b; Heinz et al. 2009; Xu et al.2012, 2016). The disruption of the Trx and GSH systems by MeHg and Hg^{2+}have been demonstrated in zebra-sea breams (Branco et al. 2011; 2012a,b) and salmon (*Salmo salar,* Bernstssen et al. 2003). MeHg can also interfere with the Trx and GSH systems in zebrafish (Yang et al. 2007; Cambier et al. 2012).

Key Event Description

In the brain, thiol (SH)- and seleno-containing proteins involved in protection against oxidative stress are mainly located in mitochondria and in the cytoplasm of the different neural cell types (Comini, 2016; Hoppe et al. 2008; Barbosa et al. 2017; Zhu et al. 2017). The main SH-containing peptide involved in protection against oxidative stress is Glutathione (GSH), a tripeptide acting as a cofactor for the enzyme peroxidase and thus serving as an indirect antioxidant donating the electrons necessary for its decomposition of H_2O_2. The seleno-containing proteins of interest are: (i) the Glutathione Peroxidase (GPx) family, involved in detoxification of hydroperoxides; (ii) the Thioredoxin Reductase (TrxR) family, involved in the regeneration of reduced thioredoxin (Pillai et al., 2014;), and the less studied SelH, K, S, R, W, and P selenoproteins (Pisoschi and Pop, 2015, Reeves and Hoffmann, 2009). Binding of chemicals to these proteins induces either their inactivation or favor their degradation (Farina et al. 2009; Zemolin et al. 2012). Of particular importance, the GSH/GPx and thioredoxin (Trx)/TrxR systems are the two main redox regulators of mammalian cells and the disruption of their activities can compromise cell viability (Ren et al. 2016).

How it is Measured or Detected

- Binding of Hg to thiol groups was analyzed by multiple collector inductively coupled plasma mass spectometry (Wiederhold et al., 2010).
- The binding affinity of methylmercury by various selenium-containing lingands was investigated by proton magnetic resonance spectometry (Sugiura et al., 1978; Arnold et al., 1986).
- A methylene blue-mediated enzyme biosensor was developed for the detection of mercury-glutathione complex. The biosensor was the enzyme horseradish peroxidase. The binding site of $HgCl_2$ with the enzyme was a cysteine residue-SH (Han et al., 2001).
- A photometric method to quantify GSH loss after reactio with organic electrophiles has also been reported (Böhme et al., 2009).
- Binding of mercuric chloride to GSH was measured by high performance liquid chromatography (HPLC)-ultraviolet (UV) detection, HPLC-inductively coupled mass spectometry and HPLC-electrospray ionization mass spectometry (Qiao et al., 2017).

- Carvalho et al. (2011) determined the binding of MeHg or Hg2+ with purified Thioredoxin Reductase using mass spectrometry. The liquid chromatography was not applied because they have used a pure chemical system, i.e, without living cells.
- Mass spectra analysis allowed to measure the binding of mercury chloride and methylmercury to proteins of the mamallian thioredixin system, thioredoxin reductase (Trx) and thioredoxin (Trx), and of the glutaredoxin system, glutathione reductase (GR) and glutaredoxin (Grx) (Carvahlo et al., 2008)
- The methodology to detect acrylamide-cysteine adducts has been performed by liquid chromatography coupled to tandem mass spectrometry (Martyniuk et al. 2013). In this paper the authors dected by using a shotgun proteomic approach a total of 15,243 peptides in ACR-exposed N27 cells. And from those 15,243 peptides, 103 peptides (from 100 different proteins) contained acrylamide-cysteine adducts.

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List of Key Events in the AOP

Event: 1538: Decreased protection against oxidative stress

Short Name: Protection against oxidative stress, decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:284 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress leads to chronic kidney disease	KeyEvent

Stressors

Name
Mercury
Acrylamide

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Male	
Female	

Glutathione, GPx and TrxR are present in bacteria, archea, algae, and in the majority of animals, including humans.

Key Event Description

High levels reactive oxygen species (ROS) can be very damaging to cells and molecules within the cell. As a result, the cell has important defense mechanisms to protect itself from ROS, such as glutathione and selenoenzymes.

Glutathione (GSH) is the most abundant low molecular mass thiol compound synthesized in cells, reaching intracellular concentrations of 1–10 mM, and is the major antioxidant and redox buffer in human cells. In fact, GSH serves as a reducing agent for ROS and other unstable molecules generated by catalytic systems, including glutathione peroxidase (GPx)(Forman, 2009).

Selenium plays a crucial role in antioxidant defense, as one Se atom is absolutely required at the active site of all selenoenzymes, such as GPx and thioredoxin reductase (TrxR), in the form of selenocystein (Rayman, 2000). GPx is an antioxidant enzyme that, in the presence of tripeptide GSH, adds two electrons to reduce H_2O_2 and lipid

peroxides to water and lipid alcohols, respectively, while simultaneously oxidizing GSH to glutathione disulfide. The GPx/GSH system is thought to be a major defense in low-level oxidative stress, and decreased GPx activity or GSH levels may lead to the absence of adequate H_2O_2 and lipid peroxides detoxification, which may be converted to OH-radicals and lipid peroxyl radicals, respectively, by transition metals (Fe^{2+}) (Brigelius-Flohe, 2013). Thioredoxin reductase (TrxR) is essential for maintaining intracellular redox status. The expression of this small (12 kDa) ubiquitous thiol-active protein is induced by ROS and an elevated serum level may indicate a state of oxidative stress. In this regard, TrxR, a NADPH-dependent lipid hydroperoxide reductase, uses NADPH to maintain the levels of reduced Trx via a mechanism similar to that used by GR to maintain GSH levels, contributing to the maintenance of thiol redox homeostasis in proteins. Importantly, the inhibition of TrxR impairs the cyclical regeneration of Trx activity, as Trx remains in the oxidized state (Bjornstedt, 1995, Zhong, 2002). Other, less studied selenoproteins, such as SelP, H, K, S, R, and W selenoproteins, play a role in antioxidant defense (Pisoschi, 2015, Reeves, 2009)

How it is Measured or Detected

- Glutathione (GSH) depletion. GSH can be measured by assaying the ratio of reduced to oxidized glutathione (GSH:GSSG) using a commercially available kit (e.g., http://www.abcam.com/gshgssg-ratio-detection-assay-kit-fluorometric-green-ab138881.html)
- Reduction of GPx activity. The activity of GPx can be measured by a colorimetric assay, using a commercially available kit (e.g., Abcam ab102530)
- Reduction of TrxR activity. The activity of TrxR can be measured by a colorimetric assay, using a commercially available kit (e.g., Abcam ab83463)
- Reduction of Selenoprotein R activity. The methionine sulfoxide reductase activity of SelR can be measured by HPLC (Chen, 2013)
- Selenoprotein P depletion. The depletion in SelP can be measured using an ELISA (e.g., MyBiosource #MBS9301054)
- Selenoprotein W depletion. The depletion in SelW can be measured using an ELISA (e.g., MyBiosource #MBS9312544)
- Selenoprotein S depletion. The depletion in SelS can be measured using an ELISA (e.g., MyBiosource #MBS9306607)
- Selenoprotein H and K depletion. The depletion in SelH and K can be measured by western blotting (Lee, 2015, Novoselov, 2007)

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Event: 1392: Oxidative Stress

Short Name: Oxidative Stress

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:220 - Cyp2E1 Activation Leading to Liver Cancer	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:284 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress leads to chronic kidney disease	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:411 - Oxidative stress Leading to Decreased Lung Function	MolecularInitiatingEvent
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	MolecularInitiatingEvent
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	MolecularInitiatingEvent
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:437 - Inhibition of mitochondrial electron transport chain (ETC) complexes leading to kidney toxicity	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent

Stressors

Name
Acetaminophen
Chloroform
furan
Platinum
Aluminum
Cadmium
Mercury

Name
Uranium
Arsenic
Silver
Manganese
Nickel
Zinc
nanoparticles

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Platinum

Kruidering et al. (1997) examined the effect of platinum on pig kidneys and found that it was able to induce significant dose-dependant ROS formation within 20 minutes of treatment administration.

Aluminum

In a study of the effects of aluminum treatment on rat kidneys, Al Dera (2016) found that renal GSH, SOD, and GPx levels were significantly lower in the treated groups, while lipid peroxidation levels were significantly increased.

Cadmium

Belyaeva et al. (2012) investigated the effect of cadmium treatment on human kidney cells. They found that cadmium was the most toxic when the sample was treated with 500 µM for 3 hours (Belyaeva et al., 2012). As this study also looked at mercury, it is worth noting that mercury was more toxic than cadmium in both 30-minute and 3-hour exposures at low concentrations (10-100 µM) (Belyaeva et al., 2012).

Wang et al. (2009) conducted a study evaluating the effects of cadmium treatment on rats and found that the treated group showed a significant increase in lipid peroxidation. They also assessed the effects of lead in this study, and found that cadmium can achieve a very similar level of lipid peroxidation at a much lower concentration than lead can, implying that cadmium is a much more toxic metal to the kidney mitochondria than lead is (Wang et al., 2009). They also found that when lead and cadmium were applied together they had an additive effect in increasing lipid peroxidation content in the renal cortex of rats (Wang et al., 2009).

Jozefczak et al. (2015) treated *Arabidopsis thaliana* wildtype, *cad2-1* mutant, and *vtc1-1* mutant plants with cadmium to determine the effects of heavy metal exposure to plant mitochondria in the roots and leaves. They found that total GSH/GSG ratios were significantly increased after cadmium exposure in the leaves of all sample varieties and that GSH content was most significantly decreased for the wildtype plant roots (Jozefczak et al., 2015).

Andjelkovic et al. (2019) also found that renal lipid peroxidation was significantly increased in rats treated with 30 mg/kg of cadmium.

Mercury

Belyaeva et al. (2012) conducted a study which looked at the effects of mercury on human kidney cells, they found that mercury was the most toxic when the sample was treated with 100 µM for 30 minutes.

Buelna-Chontal et al. (2017) investigated the effects of mercury on rat kidneys and found that treated rats had higher lipid peroxidation content and reduced cytochrome c content in their kidneys.

Uranium

In Shaki et al.'s article (2012), they found rat kidney mitochondria treated with uranyl acetate caused increased formation of ROS, increased lipid peroxidation, and decreased GSH content when exposed to 100 µM or more for an hour.

Hao et al. (2014), found that human kidney proximal tubular cells (HK-2 cells) treated with uranyl nitrate for 24 hours with 500 µM showed a 3.5 times increase in ROS production compared to the control. They also found that GSH content was decreased by 50% of the control when the cells were treated with uranyl nitrate (Hao et al., 2014).

Arsenic

Bhadauria and Flora (2007) studied the effects of arsenic treatment on rat kidneys. They found that lipid peroxidation levels were increased by 1.5 times and the GSH/GSSG ratio was decreased significantly (Bhadauria and Flora, 2007).

Kharroubi et al. (2014) also investigated the effect of arsenic treatment on rat kidneys and found that lipid peroxidation was significantly increased, while GSH content was significantly decreased.

In their study of the effects of arsenic treatment on rat kidneys, Turk et al. (2019) found that lipid peroxidation was significantly increased while GSH and GPx renal content were decreased.

Silver

Miyayama et al. (2013) investigated the effects of silver treatment on human bronchial epithelial cells and found that intracellular ROS generation was increased significantly in a dose-dependant manner when treated with 0.01 to 1.0 µM of silver nitrate.

Manganese

Chtourou et al. (2012) investigated the effects of manganese treatment on rat kidneys. They found that manganese treatment caused significant increases in ROS production, lipid peroxidation, urinary H_2O_2 levels, and PCO production. They also found that intracellular GSH content was depleted in the treated group (Chtourou et al., 2012).

Nickel

Tyagi et al. (2011) conducted a study of the effects of nickel treatment on rat kidneys. They found that the treated rats showed a significant increase in kidney lipid peroxidation and a significant decrease in GSH content in the kidney tissue (Tyagi et al., 2011).

Zinc

Yeh et al. (2011) investigated the effects of zinc treatment on rat kidneys and found that treatment with 150 µM or more for 2 weeks or more caused a time- and dose-dependant increase in lipid peroxidation. They also found that renal GSH content was decreased in the rats treated with 150 µM or more for 8 weeks (Yeh et al., 2011).

It should be noted that Hao et al. (2014) found that rat kidneys exposed to lower concentrations of zinc (such as 100 µM) for short time periods (such as 1 day), showed a protective effect against toxicity induced by other heavy metals, including uranium. Soussi, Gargouri, and El Feki (2018) also found that pre-treatment with a low concentration of zinc (10 mg/kg treatment for 15 days) protected the renal cells of rats were from changes in varying oxidative stress markers, such as lipid peroxidation, protein carbonyl, and GPx levels.

nanoparticles

Huerta-García et al. (2014) conducted a study of the effects of titanium nanoparticles on human and rat brain cells. They found that both the human and rat cells showed time-dependant increases in ROS when treated with titanium nanoparticles for 2 to 6 hours (Huerta-García et al., 2014). They also found elevated lipid peroxidation that was induced by the titanium nanoparticle treatment of human and rat cell lines in a time-dependant manner (Huerta-García et al., 2014).

Liu et al. (2010) also investigated the effects of titanium nanoparticles, however they conducted their trials on rat kidney cells. They found that ROS production was significantly increased in a dose dependant manner when treated with 10 to 100 µg/mL of titanium nanoparticles (Liu et al., 2010).

Pan et al. (2009) treated human cervix carcinoma cells with gold nanoparticles (Au1.4MS) and found that intracellular ROS content in the treated cells increased in a time-dependant manner when treated with 100 µM for 6 to 48 hours. They also compared the treatment with Au1.4MS gold nanoparticles to treatment with Au15MS treatment, which are another size of gold nanoparticle (Pan et al., 2009). The Au15MS nanoparticles were much less toxic than the Au1.4MS gold nanoparticles, even when the Au15MS nanoparticles were applied at a concentration of 1000 µM (Pan et al., 2009). When investigating further markers of oxidative stress, Pan et al. (2009) found that GSH content was greatly decreased in cells treated with gold nanoparticles.

Ferreira et al. (2015) also studied the effects of gold nanoparticles. They exposed rat kidneys to GNPs-10 (10 nm particles) and GNPs-30 (30 nm particles), and found that lipid peroxidation and protein carbonyl content in the rat kidneys treated with GNPs-30 and GNPs-10, respectively, were significantly elevated.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rodents	rodents	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Oxidative stress is produced in, and can occur in, any species from bacteria through to humans.

Key Event Description

Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses. High levels of oxidizing free radicals can be very damaging to cells and molecules within the cell. As a result, the cell has important defense mechanisms to protect itself from ROS. For example, Nrf2 is a transcription factor and master regulator of the oxidative stress response. During periods of oxidative stress, Nrf2-dependent changes in gene expression are important in regaining cellular homeostasis (Nguyen, et al. 2009) and can be used as indicators of the presence of oxidative stress in the cell.

In addition to the directly damaging actions of ROS, cellular oxidative stress also changes cellular activities on a molecular level. Redox sensitive proteins have altered physiology in the presence and absence of ROS, which is caused by the oxidation of sulfhydryls to disulfides (2SH àSS) on neighboring amino acids (Antelmann and Helmann 2011). Importantly Keap1, the negative regulator of Nrf2, is regulated in this manner (Itoh, et al. 2010).

Protection against oxidative stress is relevant for all tissues and organs, although some tissues may be more susceptible. For example, the brain possesses several key physiological features, such as high O2 utilization, high polyunsaturated fatty acids content, presence of autooxidable neurotransmitters, and low antioxidant defenses as compared to other organs, that make it highly susceptible to oxidative stress (Halliwell, 2006; Emerit and al., 2004; Frauenberger et al., 2016).

How it is Measured or Detected

Oxidative Stress. Direct measurement of ROS is difficult because ROS are unstable. The presence of ROS can be assayed indirectly by measurement of cellular antioxidants, or by ROS-dependent cellular damage:

- Detection of ROS by chemiluminescence (https://www.sciencedirect.com/science/article/abs/pii/S0165993606001683)
- Detection of ROS by chemiluminescence is also described in OECD TG 495 to assess phototoxic potential.
- Glutathione (GSH) depletion. GSH can be measured by assaying the ratio of reduced to oxidized glutathione (GSH:GSSG) using a commercially available kit (e.g., http://www.abcam.com/gshgssg-ratio-detection-assay-kit-fluorometric-green-ab138881.html).
- TBARS. Oxidative damage to lipids can be measured by assaying for lipid peroxidation using TBARS (thiobarbituric acid reactive substances) using a commercially available kit.
- 8-oxo-dG. Oxidative damage to nucleic acids can be assayed by measuring 8-oxo-dG adducts (for which there are a number of ELISA based commercially available kits),or HPLC, described in Chepelev et al. (Chepelev, et al. 2015).

Molecular Biology: Nrf2. Nrf2's transcriptional activity is controlled post-translationally by oxidation of Keap1. Assay for Nrf2 activity include:

- Immunohistochemistry for increases in Nrf2 protein levels and translocation into the nucleus
- Western blot for increased Nrf2 protein levels
- Western blot of cytoplasmic and nuclear fractions to observe translocation of Nrf2 protein from the cytoplasm to the nucleus
- qPCR of Nrf2 target genes (e.g., Nqo1, Hmox-1, Gcl, Gst, Prx, TrxR, Srxn), or by commercially available pathway-based qPCR array (e.g., oxidative stress array from

SABiosciences)
- Whole transcriptome profiling by microarray or RNA-seq followed by pathway analysis (in IPA, DAVID, metacore, etc.) for enrichment of the Nrf2 oxidative stress response pathway (e.g., Jackson et al. 2014)
- OECD TG422D describes an ARE-Nrf2 Luciferase test method
- In general, there are a variety of commercially available colorimetric or fluorescent kits for detecting Nrf2 activation

Assay Type & Measured Content	Description	Dose Range Studied	Assay Characteristics (Length / Ease of use/Accuracy)
ROS Formation in the Mitochondria assay (Shaki et al., 2012)	"The mitochondrial ROS measurement was performed flow cytometry using DCFH-DA. Briefly, isolated kidney mitochondria were incubated with UA (0, 50, 100 and 200 µM) in respiration buffer containing (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 µM EGTA, 0.5 mM MgCl2, 0.1 mM KH2PO4 and 5 mM sodium succinate) [32]. In the interval times of 5, 30 and 60 min following the UA addition, a sample was taken and DCFH-DA was added (final concentration, 10 µM) to mitochondria and was then incubated for 10 min. Uranyl acetate-induced ROS generation in isolated kidney mitochondria were determined through the flow cytometry (Partec, Deutschland) equipped with a 488-nm argon ion laser and supplied with the Flomax software and the signals were obtained using a 530-nm bandpass filter (FL-1 channel). Each determination is based on the mean fluorescence intensity of 15,000 counts."	0, 50, 100 and 200 µM of Uranyl Acetate	Long/ Easy High accuracy
Mitochondrial Antioxidant Content Assay Measuring GSH content (Shaki et al., 2012)	"GSH content was determined using DTNB as the indicator and spectrophotometer method for the isolated mitochondria. The mitochondrial fractions (0.5 mg protein/ml) were incubated with various concentrations of uranyl acetate for 1 h at 30 °C and then 0.1 ml of mitochondrial fractions was added into 0.1 mol/l of phosphate buffers and 0.04% DTNB in a total volume of 3.0 ml (pH 7.4). The developed yellow color was read at 412 nm on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was expressed as µg/mg protein."	0, 50, 100, or 200 μM Uranyl Acetate	
H_2O_2 Production Assay Measuring H_2O_2 Production in isolated mitochondria (Heyno et al., 2008)	"Effect of $CdCl_2$ and antimycin A (AA) on H_2O_2 production in isolated mitochondria from potato. H_2O_2 production was measured as scopoletin oxidation. Mitochondria were incubated for 30 min in the measuring buffer (see the Materials and Methods) containing 0.5 mM succinate as an electron donor and 0.2 µM mesoxalonitrile 3-chlorophenylhydrazone (CCCP) as an uncoupler, 10 U horseradish peroxidase and 5 µM scopoletin." (	0, 10, 30 μM Cd^{2+} 2 μM antimycin A	
Flow Cytometry ROS & Cell Viability (Kruiderig et al., 1997)	"For determination of ROS, samples taken at the indicated time points were directly transferred to FACScan tubes. Dih123 (10 mM, final concentration) was added and cells were incubated at 37°C in a humidified atmosphere (95% air/5% CO2) for 10 min. At *t* 5 9, propidium iodide (10 mM, final concentration) was added, and cells were analyzed by flow cytometry at 60 ml/min. Nonfluorescent Dih123 is cleaved by ROS to fluorescent R123 and detected by the FL1 detector as described above for Dc (Van de Water 1995)"		Strong/easy medium
DCFH-DA Assay Detection of hydrogen peroxide production (Yuan et al., 2016)	Intracellular ROS production was measured using DCFH-DA as a probe. Hydrogen peroxide oxidizes DCFH to DCF. The probe is hydrolyzed intracellularly to DCFH carboxylate anion. No direct reaction with H_2O_2 to form fluorescent production.	0-400 µM	Long/ Easy High accuracy
H2-DCF-DA Assay Detection of superoxide production (Thiebault et al., 2007)	This dye is a stable nonpolar compound which diffuses readily into the cells and yields H2-DCF. Intracellular OH or ONOO- react with H2-DCF when cells contain peroxides, to form the highly fluorescent compound DCF, which effluxes the cell. Fluorescence intensity of DCF is measured using a fluorescence spectrophotometer.	0–600 µM	Long/ Easy High accuracy
CM-H2DCFDA Assay	**Come back and explain the flow cytometry determination of oxidative stress from Pan et al. (2009)**		

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Event: 1488: Glutamate dyshomeostasis

Short Name: Glutamate dyshomeostasis

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

neural cell

Organ term

Organ term

brain

Domain of Applicability

The involvement of glutamate in learning and memory processes is well conserved in all taxa, from invertebrates (ex. Drosophila) to vertebrates (Fagnou and Tuchek, 1995).

Key Event Description

Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), where it plays major roles in multiple aspects, such as development, learning, memory and response to injury (Featherstone, 2010). However, it is well recognized that Glu at high concentrations at the synaptic cleft acts as a toxin, inducing neuronal injury and death (Meldrum, 2000; Ozawa et al., 1998) secondary to activation of glumatergic *N*-methyl D-aspartate (NMDA) receptors and Ca^{2+} influx. Glu dyshomeostasis is a consequence of perturbation of astrocyte/neuron interactions and the transport of this amino acid, as will be discussed below.

Astrocytes are critically involved in neuronal function and survival, as they produce neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glia-derived neurotrophic factor (GDNF), as well as express two main glutamate transporters responsible for the removal of excessive Glu from the synaptic clefts (Chai et al., 2013; Sheldon et al., 2007). Glutamate is the major excitatory neurotransmitter in the CNS, playing a major role in memory and cognitive function (Platt, 1997), and Glu transporters as such prevent the overstimulation of post-synaptic glutamate receptors that lead to excitotoxic neuronal injury (Sattler et al., 2001; Dobble, 1999). Among the five subtypes of Glu transporters identified, glutamate aspartate transporter (GLAST) and Glu transporter-1 (GLT-1) [excitatory amino acid transporter (EAAT) 1 and 2 in humans, respectively], are predominantly expressed in astrocytes. They are responsible for the uptake of excess glutamate from the extracellular space (Furuta et al., 1997; Lehre et al., 1995; Tanaka, 2000), supported by the fact that knockdown of either GLT-1 or GLAST in mice increases extracellular glutamate levels, leading to excitotoxicity related neurodegeneration and progressive paralysis (Bristol and Rothstein, 1996). In the adult brain, EAAT2 accounts for >90% of extracellular glutamate clearance (Danbolt, 2001; Kim et al., 2011; Rothstein et al., 1995), and genetic deletion of both alleles of GLT-1 in mice leads to the development of lethal seizures (Rothstein et al., 1996). On the other hand, EAAT1-3 play a major role during human brain development, in particular in corticogenesis, where they are expressed in proliferative zones and in radial glia, and alterations of Glu transporters contributes to disorganized cortex seen in migration disorders (Furuta et al., 2005;Regan et al., 2007). Indeed, disruption of glutamate signaling is thought to be part of the etiology underlying some neurodevelopmental disorders such as autism and schizophrenia (Chiocchetti et al., 2014; Schwartz et al., 2012). Genes involved in glutamatergic pathways, affecting receptor signalling, metabolism and transport, were enriched in genetic variants associated with autism spectrum disorders (Chiocchetti et al, 2014).

Extracellular Glu released by neurons is taken up by astrocytes, which is converted into glutamine (Gln) by glutamine synthetase (GS), a thiol-containing enzyme (cf MIE, Binding to SH-/seleno containing proteins). Intercellular compartmentation of Gln and Glu, the so-called Gln/Glu-GABA cycle (GGC), is critical for optimal CNS function.13C NMR studies have demonstrated that the ratio of Gln/Glu is extremely high and increases with brain activity (Shen et al., 1999). Thus the GGC gives rise to the amino acid neurotransmitters Glu and GABA via dynamic astrocyte neuron interactions. Glu released at synaptic terminals is taken up by surrounding astrocytes via GLT-1 and GLAST (Rothstein et al., 1994; 1996). A small proportion of the astrocytic formed Gln via a reaction mediated by GS is transported into the extracellular space by Gln carriers, with a predominant role for System N/A transporter (SNAT3), which belongs to the bidirectional transporter System N (Chaudhry et al., 2002).

In addition to System N, release of Gln from astrocytes is mediated by other transport systems, including Systems L (LAT2) and ASC (ASCT2). Extracellular Gln is taken up into GABAergic and Glu-ergic neurons by the unidirectional System A transporters SNAT1 (Melone et al., 2004) and SNAT2 (Grewal et al., 2009). Once in neurons, Gln is converted to Glu by the mitochondrial enzyme phosphate-activated glutaminase (Kvamme et al., 2001). Additionally, Glu is packaged into synaptic vesicles by the vesicular VGLUT transporter (Bellocchio et al., 1998), released into the extracellular space and taken up by astrocytes where it is converted back to Gln by GS, thus completing the GGC (Fig. 1).

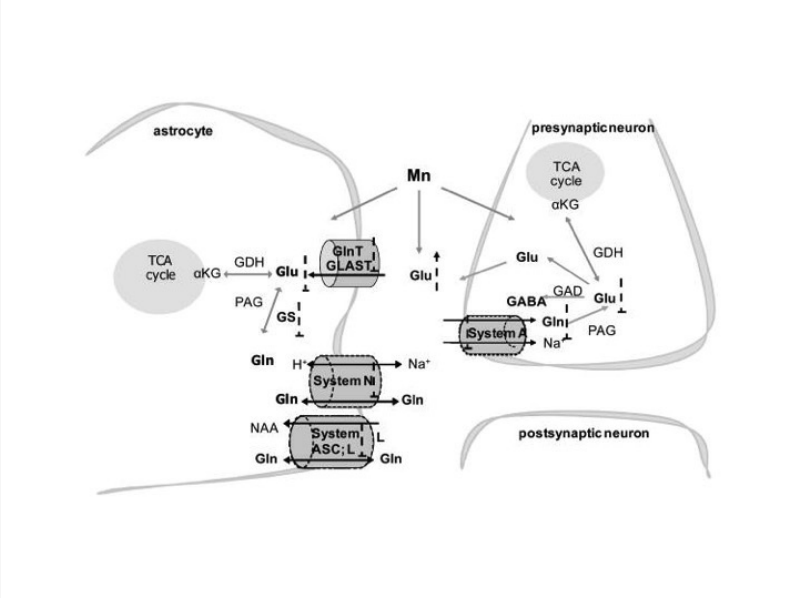

Figure 1: Schematic representation of Glu and Gln transport systems related to the GGC. From Sidorik-Wegrzynowicz and Aschner, 2013)

How it is Measured or Detected

L-Glu transporter activities can be quantified by direct or indirect methods:

- Direct quantification, L-Glu transporter activities are determined by the amount of ^{3}H-labeled ligand (L-Glu or D-aspartate) taken up by the cells (Primary mixed astrocyte and neuron cultures [Perez-Dominguez et al., 2014]; primary astrocyte cultures [Matos et al., 2008; Li et al., 2006; Hazell et al., 2003]; Xenopus laevis oocyte overexpressing the L-Glu transporter subtype of interest [Sogaard et al., 2013; Trotti et al., 2001]; transfected HeLa cells [Zhang and Qu, 2012]) or tissues (ex. Hippocampal tissue [Selkirk et al., 2005])
- Indirect quantification, L-Glu transporter activities are determined by the L-Glu residue in the medium or buffer after incubation with cells expressing the different L-Glu transporters (Brison et al., 2014; Xin et al., 2019; Jin et al., 2015; Gu et al., 2014; Abe et alt.2000], .).
- The transport activity of the different L-Glu transporter subtypes should be determined in the presence of the appropriate inhibitors as shown in the table 1. Ex: The glutamate uptake activity via EAAT1 can be determined in the presence of dihydrokainic acid (DHK), a specific inhibitor for GLT-1, as described in Mutkus et al. (2005). Expression level of L-Glu transporter subtypes should be confirmed using Western blotting or immunocytochemistry. It is interesting to note that pure astrocyte culture express only GLAST (EAAT1) (Danbolt et al., 2016); whereas In mixed astrocyte and neuron cultures, GLAST (EAAT1) and GLT-1(EAAT2) are expressed (Danbolt et al., 2016). The expression of GLT1 (EAAT2) is suggested to be induced by soluble factors (Gegelashvili et al., 1997, 2000; Plachez et al., 2000; Martinez-Lozada et al., 2016).
- The L-Glu concentrations in medium or in incubation buffer can be quantified by commercially-available kits quantifying the final products of the redox reaction in which L-Glu is a substrate.

The kits using colorimetric final products (OD=450 nm):

- Glutamate Assay Kit from Abcam (ab83389)
- Glutamate Colorimetric Assay Kit from BioVision (K629)
- Glutamate Assay Kit from Merck (MAK004)

The kit using the bioluminescent metabolite:

- Glutamate-Glo™ Assay from Promega (J7021)

Table 1: Summary based on the reviews of Murphy-Royal et al., 2017 and Pajarillo et al., 2019, with some modification. Concerning the physiological functions of each EAAT subtype, see the review by Danbolt (Danbolt, 2001).

Human	Rodent	Distribution	Non-specific inhibitors	Specific inhibitors
EAAT1	GLAST	High expression in astrocytes at developmental stage	DL-threo-b-benzyloxyaspartate (TBOA) and its variants (e.g. PMBTBOA and TFB-TBOA) (Bridges et al.,1999; Lebrun et al., 1997; Shimamoto et al., 1998; Shimamoto, 2008)	UCPH101 (Erichsen et al., 2010)
EAAT2	GLT-1	Astrocytes (>90% adult CNS L-Glu uptake) Neuronal terminals (hippocampus, cortex, still controversial)		WAY213613 (Dunlop et al., 2005) DHK (Arriza et al., 1994; Bridges et al., 1999
EAAT3	EAAC1	Neurons. Especially high in hippocampal neurons. Also function as Cys transporter (Watts et al., 2014)		
EAAT4	EAAT4	Purkinje cells in the cerebellum		
EAAT5	EAAT5	Retina. Very weak in the CNS		

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Event: 55: Cell injury/death

Short Name: Cell injury/death

Key Event Component

Process	Object	Action
cell death		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent
Aop:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent

AOP ID and Name	Event Type
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:278 - IKK complex inhibition leading to liver injury	KeyEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

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

Key Event Description

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

DNA damage activates nuclear poly(ADP-ribose) polymerase-1(PARP-1), a DNA repair enzyme. PARP-1 forms poly(ADP-ribose) polymers, to repair DNA, but when DNA damage is extensive, PAR accumulates, exits cell nuclei and travels to mitochondrial membranes, where it, like calpain I, is involved in AIF release from mitochondria. A fundamental distinction between necrosis and apoptosis is the loss of plasma membrane integrity; this is integral to the former but not the latter. As a consequence, lytic release of cellular constituents promotes a local inflammatory reaction, whereas the rapid removal of apoptotic bodies minimizes such a reaction. The distinction between the two modes of death is easily accomplished in vitro but not in vivo. Thus, although claims that certain drugs induce apoptosis have been made, these are relatively unconvincing. DNA fragmentation can occur in necrosis, leading to positive TUNEL staining (see explanation below). Conversely, when apoptosis is massive, it can exceed the capacity for rapid phagocytosis, resulting in the eventual appearance of secondary necrosis.

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

How it is Measured or Detected

Necrosis:

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

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

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

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

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

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

Apoptosis:

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

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

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

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

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Event: 188: Neuroinflammation

Short Name: Neuroinflammation

Key Event Component

Process	Object	Action
brain inflammation	microglial cell	pathological
brain inflammation	astrocyte	pathological

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	KeyEvent
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:3 - Inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits	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:374 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on brain cells (neuronal and non-neuronal) leads to neuroinflammation resulting in encephalitis	KeyEvent
Aop: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:464 - Calcium overload in dopaminergic neurons of the substantia nigra leading to parkinsonian motor deficits	KeyEvent

Stressors

Name
SARS-CoV
Sars-CoV-2
Chemical
Virus
bacteria

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Mixed	High

Neuroinflammation is observed in human, monkey, rat, mouse, and zebrafish, in association with neurodegeneration or following toxicant exposure, or SARS-CoV-2 and other coronavirus infection. Some references (non-exhaustive list) are given below for illustration:

Human: Vennetti et al., 2006

Monkey (Macaca fascicularis): Charleston et al., 1994, 1996

Rat: Little et al., 2012; Zurich et al., 2002; Eskes et al., 2002

Mouse: Liu et al., 2012

Zebrafish: Xu et al., 2014.

Key Event Description

Neuroinflammation or brain inflammation differs from peripheral inflammation in that the vascular response and the role of peripheral bone marrow-derived cells are less conspicuous. The most easily detectable feature of neuroinflammation is activation of microglial cells and astrocytes. It is evidenced by changes in shape, increased expression of certain antigens, and accumulation and proliferation of the glial cells in affected regions (Aschner, 1998; Graeber & Streit, 1990; Monnet-Tschudi et al, 2007; Streit et al, 1999; Kraft and Harry, 2011; Claycomb et al., 2013). Upon stimulation by cytokines or inflammogens (e.g. from pathogens or from damaged neurons), both glial cell types activate inflammatory signalling pathways, which result in increased expression and/or release of inflammatory mediators such as cytokines, eicosanoids, and metalloproteinases (Dong & Benveniste, 2001), as well as in the production of reactive oxygen (ROS) and nitrogen species (RNS) (Brown & Bal-Price, 2003). Different types of activation states are possible for microglia and astrocytes, resulting in pro-inflammatory or anti-inflammatory signalling and other cellular functions (such as phagocytosis) (Streit et al., 1999; Nakajima and Kohsaka, 2004).

Therefore, neuroinflammation can have both neuroprotective/neuroreparative and neurodegenerative consequences (Carson et al., 2006 ; Monnet-Tschudi et al, 2007; Aguzzi et al., 2013 ; Glass et al., 2010). Under normal physiological conditions, microglial cells scan the nervous system for neuronal integrity (Nimmerjahn et al, 2005) and for invading pathogens (Aloisi, 2001; Kreutzberg, 1995; Kreutzberg, 1996; Rivest, 2009). They are the first type of cell activated (first line of defence), and can subsequently induce astrocyte activation (Falsig, 2008). Two distinct states of microglial activation have been described (Gordon, 2003; Kigerl et al, 2009; Maresz et al, 2008; Mosser &

Edwards, 2008; Perego et al; Ponomarev et al, 2005; Moehle and West, 2015): The M1 state is classically triggered by interferon-gamma and/or other pro-inflammatory cytokines, and this state is characterized by increased expression of integrin alpha M (Itgam) and CD86, as well as the release of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6), and it is mostly associated with neurodegeneration. The M2 state is triggered by IL-4 and IL-13 (Maresz et al, 2008; Perego et al, 2011; Ponomarev et al, 2007) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes. The activation of astrocytes by microglia-derived cytokines or TLR agonists resembles the microglial M1 state (Falsig 2006). Although classification of the M1/M2 polarization of microglial cells may be considered as a simplification of authentic microglial reaction states (Ransohoff, 2016), a similar polarization of reactive astrocytes has been descibed recently Liddlelow et al., 2017): Interleukin-1 alpha (IL-1alpha), TNF and subcomponent q (C1q) released by activated microglial cells induce A1-reactive astrocytes, which lose the ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis and induce the death of neurons and oligodendrocytes.

Neuroinflammation and Brain development

During brain development, microglia are known to play a critical role as shapers of neural circuits, by providing trophic factors and by remodeling and pruning synapses (Rajendran and Paolicelli, 2018). In addition to playing a role in synaptic management, microglia are important for the pruning of dying neurons and in the clearance of debris (Harry, 2013). Microglia seem to affect also processes associated with neuronal proliferation and differentiation (Harry and Kraft, 2012). Similarly to microglia, astrocytes have instructive roles in neurogenesis, gliogenesis, angiogenesis, axonal outgrowth, synaptogenesis, and synaptic pruning (Reemst et al., 2016).

The development-dependent reactivity of microglial cells and astrocytes is not well known. Ischemic insult appears to elicit similar microglial reactivity both during brain development and in adulthood (Baburamani et al, 2014; Leonardo & Pennypacker, 2009). In contrast, treatment with lead acetate was previously shown to result in a more pronounced microglial and astrocyte reactivity in immature 3D rat brain cell cultures as compared to mature ones (Zurich et al, 2002). Astrocyte reactivity was also more pronounced in immature 3D rat brain cell cultures following paraquat exposure, whereas development-dependent differences in the phenotype of reactive microglia were observed (Sandström et al., 2017). This suggests that neuroinflammation is occurring during brain development and may express a different phenotype than in adulthood, and that dysfunction of microglia and astrocyte during brain development could contribute to neurodevelopmental disorders and potentially to late-onset neuropathology (Reemst et al., 2016).

Neuroinflammation in relation to COVID19

SARS-CoV-2 patients with moderate and severe COVID-19 presented an elevated plasma levels of glial fibrillary acidic protein (GFAP), which is known as biochemical indicator of glial activation (Kanberg et al., 2020).

How it is Measured or Detected

Neuroinflammation, i.e. the activation of glial cells can be measured by quantification of cellular markers (most commonly), or of released mediators (less common). As multiple activation states exist for the two main cell types involved, it is necessary to measure several markers of neuroinflammation:

- Microglial activation can be detected based on the increased numbers of labeled microglia per volume element of brain tissue (due to increase of binding sites, proliferation, and immigration of cells) or on morphological changes. A specific microglial marker, used across different species, is CD11b. Alternatively various specific carbohydrate structures can be stained by lectins (e.g. IB4). Beyond that, various well-established antibodies are available to detect microglia in mouse tissue (F4/80), phagocytic microglia in rat tissue (ED1) or more generally microglia across species (Iba1). Transgenic mice are available with fluorescent proteins under the control of the CD11b promoter to easily quantify microglia without the need for specific stains.
- The most frequently used astrocyte marker is GFAP (99% of all studies) (Eng et al., 2000). This protein is highly specific for astrocytes in the brain, and antibodies are available for immunocytochemical detection. In neuroinflammatory brain regions, the stain becomes more prominent, due to an upregulation of the protein, a shape change/proliferation of the cells, and/or better accessibility of the antibody. Various histological quantification approaches can be used. Occasionally, alternative astrocytic markers, such as vimentin of the S100beta protein, have been used for staining of astrocytes (Struzynska et al., 2007). Antibodies for complement component 3 (C3), the most characteristic and highly upregulated marker of A1 neurotoxic reactive astrocytes are commercially available.
- All immunocytochemical methods can also be applied to cell culture models.
- In patients, microglial accumulation can be monitored by PET imaging, using [11C]-PK 11195 as a microglial marker (Banati et al., 2002).
- Activation of glial cells can be assessed in tissue or cell culture models also by quantification of sets of activation markers. This can for instance be done by PCR quantification of inflammatory factors, by measurement of the respective mediators, e.g. by ELISA-related immuno-quantification. Such markers include:
- Pro- and anti-inflammatory cytokine expression (IL-1β; TNF-α, Il-6, IL-4); or expression of immunostimmulatory proteins (e.g. MHC-II)
- Itgam, CD86 expression as markers of M1 microglial phenotype
- Arg1, MRC1, as markers of M2 microglial phenotype

For descriptions of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014

Regulatory example using the KE

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

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Event: 1492: Tissue resident cell activation

Short Name: Tissue resident cell activation

Key Event Component

Process	Object	Action
cell activation involved in immune response		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Extend to at least invertebrates

Not to plants and not to single-celled organisms

BRAIN:

Tissue resident activation is observed in human, monkey, rat, mouse, and zebrafish, in association with neurodegeneration or following toxicant exposure. Some references (non-exhaustive list) are given below for illustration:

Human: Vennetti et al., 2006

Monkey (Macaca fascicularis): Charleston et al., 1994, 1996

Rat: Little et al., 2012; Zurich et al., 2002; Eskes et al., 2002

Mouse: Liu et al., 2012

Zebrafish: Xu et al., 2014.

LIVER:

Human: Su et al., 2002; Kegel et al., 2015; Boltjes et al.,2014

Rat: Luckey and Peterson,2001

Mouse: Dalton t al., 2009

Key Event Description

Tissue resident cell activation is considered as a hallmark of inflammation irrespective of the tissue type. Strategically placed cells within tissues respond to noxious stimuli, thus regulating the recruitment of neutrophil and the initiation and resolution of inflammation (Kim and Luster, 2015). Examples for these cells are resident immune cells, parenchymal cells, vascular cells, stromal cells, or smooth muscle cells. These cells may be specific for a certain tissue, but they have a common tissue-independent role.

Under healthy conditions there is a homeostatic state, characterized as a generally quiescent cellular milieu. Various danger signals or alarmins that are involved in induction of inflammation like pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) activate these resident cells in affected tissues.

Examples of well-characterized DAMPs (danger signals or alarmins) (Saïd-Sadier and Ojcius, 2012)

DAMPs	Receptors	Outcome of receptor ligation
Extracellular nucleotides (ATP, ADP, adenosine)	PI, P2X and P2Y receptors (ATP, ADP); Al, A2A, A2B and A3 receptors (adenosine)	Dendritic cell (DC) maturation, chemotaxis, secretion of cytokines (IL-1β, IL-18), inflammation
Extracellular heat shock proteins	CD14, CD91, scavenger receptors, TLR4, TLR2, CD40	DC maturation, cytokine induction, DC, migration to lymph nodes
Extracellular HMGB1	RAGE, TLR2, TLR4	Chemotaxis, cytokine induction, DC activation, neutrophil recruitment, inflammation, activation of immune cells
Uric acid crystals	CD14, TLR2, TLR4	DC activation, cytokine induction, neutrophil recruitment, gout induction
Oxidative stress	Intracellular redox-	Cell death, release of endogenous

DAMPs	Receptors	Downstream inflammatory consequences
Laminin	integrins	Neutrophil recruitment, phagocytosis
S100 proteins or calgranulins	RAGE	Neutrophil recruitment, chemotaxis, cytokine secretion, apoptosis
Hyaluronan	TLR2, TLR4, CD44	DC maturation, cytokine production, adjuvant activity

Activation refers to a phenotypic modification of the resident cells that includes alterations in their secretions, activation of biosynthetic pathways, production of pro-inflammatory proteins and lipids, and morphological changes. While these represent a pleiotropic range of responses that can vary with the tissue, there are a number of common markers or signs of activation that are measurable.

Examples of Common markers are

- NF-kB
- AP-1
- Jnk
- P38/mapk

These described commonalities allow the use of this KE as a hub KE in the AOP network. However, despite the similarities in the inflammatory process, the type of reactive cells and the molecules triggering their reactivity may be tissue-specific. Therefore, for practical reasons, a tissue specific description of the reactive cells and of the triggering factors is necessary in order to specify in a tissue-specific manner, which cell should be considered and what should be measured.

BRAIN

The most easily detectable feature of brain inflammation or neuroinflammation is activation of microglial cells and astrocytes. It is evidenced by changes in shape, increased expression of certain antigens, and accumulation and proliferation of the glial cells in affected regions (Aschner, 1998; Graeber & Streit, 1990; Monnet-Tschudi et al, 2007; Streit et al, 1999; Kraft and Harry, 2011; Claycomb et al., 2013). Upon stimulation by cytokines, chemokines or inflammogens (e.g. from pathogens or from damaged neurons), both glial cell types activate inflammatory signaling pathways, which result in increased expression and/or release of inflammatory mediators such as cytokines, eicosanoids, and metalloproteinases (Dong & Benveniste, 2001) (cf KE: pro-inflammatory mediators, increased), as well as in the production of reactive oxygen species (ROS) and nitrogen species (RNS) (Brown & Bal-Price, 2003). Different types of activation states are possible for microglia and astrocytes, resulting in pro-inflammatory or anti-inflammatory signaling, and other cellular functions (such as phagocytosis) (Streit et al., 1999; Nakajima and Kohsaka, 2004). Therefore, neuroinflammation can have both neuroprotective/neuroreparative and neurodegenerative consequences (Carson et al., 2006; Monnet-Tschudi et al, 2007; Aguzzi et al., 2013 ; Glass et al., 2010). Under normal physiological conditions, microglial cells survey the nervous system for neuronal integrity (Nimmerjahn et al, 2005) and for invading pathogens (Aloisi, 2001; Kreutzberg, 1995; Kreutzberg, 1996; Rivest, 2009). They are the first type of cell activated (first line of defense), and can subsequently induce astrocyte activation (Falsig, 2008). Two distinct states of microglial activation have been described (Gordon, 2003; Kigerl et al, 2009; Maresz et al, 2008; Mosser & Edwards, 2008; Perego et al; Ponomarev et al, 2005): The M1 state is classically triggered by interferon-gamma and/or other pro-inflammatory cytokines, and this state is characterized by increased expression of integrin alpha M (Itgam) and CD86, as well as the release of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6), and it is mostly associated with neurodegeneration. The M2 state is triggered by IL-4 and IL-13 (Maresz et al, 2008; Perego et al, 2011; Ponomarev et al, 2007) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes. The activation of astrocytes by microglia-derived cytokines or TLR agonists resembles the microglial M1 state (Falsig 2006). Although classification of the M1/M2 polarization of microglial cells may be considered as a simplification of authentic microglial reaction states (Ransohoff, 2016), a similar polarization of reactive astrocytes has been described recently Liddlelow et al., 2017): Interleukin-1 alpha (IL-1a), TNF and subcomponent q (C1q) released by activated microglial cells induce A1-reactive astrocytes, which lose the ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis and induce the death of neurons and oligodendrocytes.

Regulatory examples using the KE

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

LIVER:

Kupffer cells (KCs) are a specialized population of macrophages that reside in the liver; they were first described by Carl Wilhelm von Kupffer (1829–1902) [Haubrich 2004]. KCs constitute 80%-90% of the tissue macrophages in the reticuloendothelial system and account for approximately 15% of the total liver cell population [Bouwens et al., 1986]. They play an important role in normal physiology and homeostasis as well as participating in the acute and chronic responses of the liver to toxic compounds. Activation of KCs results in the release of an array of inflammatory mediators, growth factors, and reactive oxygen species. This activation appears to modulate acute hepatocyte injury as well as chronic liver responses including hepatic cancer. Understanding the role KCs play in these diverse responses is key to understanding mechanisms of liver injury [Roberts et al.,2007]. Besides the release of inflammatory mediators including cytokines, chemokines, lysosomal and proteolytic enzymes KCs are a main source of TGF-β1 (transforming growth factor-beta 1, the most potent profibrogenic cytokine). In addition latent TGF-β1 can be activated by KC-secreted matrix metalloproteinase 9 (MMP-9)[Winwood and Arthur, 1993; Luckey and Peeterson, 2001] through the release of biologically active substances that promote the pathogenic process. Activated KCs also release ROS like superoxide generated by NOX (NADPH oxidase), thus contributing to oxidative stress. Oxidative stress also activates a variety of transcription factors like NF-κB, PPAR-γ leading to an increased gene expression for the production of growth factors, inflammatory cytokines and chemokines. KCs express TNF-α (Tumor Necrosis Factor-alpha), IL-1 (Interleukin-1) and MCP-1 (monocyte-chemoattractant protein-1), all being mitogens and chemoattractants for hepatic stellate cells (HSCs) and induce the expression of PDGF receptors on HSCs which enhances cell proliferation. Expressed TNF-α, TRAIL (TNF-related apoptosis-inducing ligand), and FasL (Fas Ligand) are not only pro-inflammatory active but also capable of inducing death receptor-mediated apoptosis in hepatocytes [Guo and Friedman, 2007; Friedman 2002; Roberts et al., 2007]. Under conditions of oxidative stress macrophages are further activated which leads to a more enhanced inflammatory response that again further activates KCs though cytokines (Interferon gamma (IFNγ), granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α), bacterial lipopolysaccharides, extracellular matrix proteins, and other chemical mediators [Kolios et al., 2006; Kershenobich Stalnikowitz and Weissbrod 2003].

Besides KCs, the resident hepatic macrophages, infiltrating bone marrow-derived macrophages, originating from circulating monocytes are recruited to the injured liver via chemokine signals. KCs appear essential for sensing tissue injury and initiating inflammatory responses, while infiltrating Ly-6C+ monocyte-derived macrophages are linked to chronic inflammation and fibrogenesis. The profibrotic functions of KCs (HSC activation via paracrine mechanisms) during chronic hepatic injury remain functionally relevant, even if the infiltration of additional inflammatory monocytes is blocked via pharmacological inhibition of the chemokine CCL2 [Baeck et al., 2012; Tacke and Zimmermann, 2014].

KC activation and macrophage recruitment are two separate events and both are necessary for fibrogenesis, but as they occur in parallel, they can be summarised as one KE.

Probably there is a threshold of KC activation and release above which liver damage is induced. Pre-treatment with gadolinium chloride (GdCl), which inhibits KC function, reduced both hepatocyte and sinusoidal epithelial cell injury, as well as decreased the numbers of macrophages appearing in hepatic lesions and inhibited TGF-β1 mRNA expression in macrophages. Experimental inhibition of KC function or depletion of KCs appeared to protect against chemical-induced liver injury [Ide et al.,2005].

How it is Measured or Detected

In General:

Measurement targets are cell surface and intracellular markers; the specific markers may be cell and species-specific.

Available methods include cytometry, immunohistochemistry, gene expression sequencing; western blotting, ELISA, and functional assays.

BRAIN

Neuroinflammation, i.e. the activation of glial cells can be measured by quantification of cellular markers (most commonly), or of released mediators (less common). As

multiple activation states exist for the two main cell types involved, it is necessary to measure several markers of neuroinflammation:

1. Microglial activation can be detected based on the increased numbers of labeled microglia per volume element of brain tissue (due to increase of binding sites, proliferation, and immigration of cells) or on morphological changes. A specific microglial marker, used across different species, is CD11b. Alternatively various specific carbohydrate structures can be stained by lectins (e.g. IB4). Beyond that, various well-established antibodies are available to detect microglia in mouse tissue (F4/80), phagocytic microglia in rat tissue (ED1) or more generally microglia across species (Iba1). Transgenic mice are available with fluorescent proteins under the control of the CD11b promoter to easily quantify microglia without the need for specific stains.
2. The most frequently used astrocyte marker is glial fibrillary acidic protein, GFAP (99% of all studies) (Eng et al., 2000). This protein is highly specific for astrocytes in the brain, and antibodies are available for immunocytochemical detection. In neuroinflamatory brain regions, the stain becomes more prominent, due to an upregulation of the protein, a shape change/proliferation of the cells, and/or better accessibility of the antibody. Various histological quantification approaches can be used. Occasionally, alternative astrocytic markers, such as vimentin of the S100beta protein, have been used for astrocyte staining (Struzynska et al., 2007). Antibodies for complement component 3 (C3), the most characteristic and highly upregulated marker of A1 neurotoxic reactive astrocytes are commercially available.
3. All immunocytochemical methods can also be applied to cell culture models.
4. In patients, microglial accumulation can be monitored by PET imaging, using [11C]-PK 11195 as a microglial marker (Banati et al., 2002).
5. Activation of glial cells can be assessed in tissue or cell culture models also by quantification of sets of M1/M2 phenotype markers. This can for instance be done by PCR quantification, immunocytochemistry, immunoblotting.

- Itgam, CD86 expression as markers of M1 microglial phenotype
- Arg1, MRC1, as markers of M2 microglial phenotype

(for descriptions of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014)

LIVER:

Kupffer cell activation can be measured by means of expressed cytokines, e.g. tissue levels of TNF-a [Vajdova et al,2004], IL-6 expression, measured by immunoassays or Elisa (offered by various companies), soluble CD163 [Grønbaek etal., 2012; Møller etal.,2012] or increase in expression of Kupffer cell marker genes such as Lyz, Gzmb, and Il1b, (Genome U34A Array, Affymetrix); [Takahara et al.,2006]

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Event: 1493: Increased Pro-inflammatory mediators

Short Name: Increased pro-inflammatory mediators

Key Event Component

Process	Object	Action
acute inflammatory response		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:432 - Deposition of Energy by Ionizing Radiation leading to Acute Myeloid Leukemia	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

LIVER:

Human [Santibañez et al., 2011]

Rat [Luckey and Petersen, 2001]

Mouse [Nan et al., 2013]

BRAIN:

Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014

Key Event Description

Inflammatory mediators are soluble, diffusible molecules that act locally at the site of tissue damage and infection, and at more distant sites. They can be divided into exogenous and endogenous mediators.

Exogenous mediators of inflammation are bacterial products or toxins like endotoxin or LPS. Endogenous mediators of inflammation are produced from within the (innate and adaptive) immune system itself, as well as other systems. They can be derived from molecules that are normally present in the plasma in an inactive form, such as peptide fragments of some components of complement, coagulation, and kinin systems. Or they can be released at the site of injury by a number of cell types that either contain them as preformed molecules within storage granules, e.g. histamine, or which can rapidly switch on the machinery required to synthesize the mediators.

Table1: a non-exhaustive list of examples for pro-inflammatory mediators

Classes of inflammatory mediators	Examples
Pro-inflammatory cytokines	TNF-a, Interleukins (IL-1, IL-6, IL-8), Interferons (IFN-g), chemokines (CXCL, CCL, GRO-α, MCP-1), GM-CSF
Prostaglandins	PGE2
Bradykinin	
Vasoactive amines	histamine, serotonin
Reactive oxygen species (ROS)	O^{2-}, H_2O_2

[illegible]	[illegible]
Reactive nitrogen species (RNS)	NO, iNOS

The increased production of pro-inflammatory mediators can have negative consequences on the parenchymal cells leading even to cell death, as described for TNF-a or peroxynitrite on neurons (Chao et al., 1995; Brown and Bal-Price, 2003). In addition, via a feedback loop, they can act on the reactive resident cells thus maintaining or exacerbating their reactive state; and by modifying elements of their signalling pathways, they can favour the M1 phenotypic polarization and the chronicity of the inflammatory process (Taetzsch et al., 2015).

Basically, this event occurs equally in various tissues and does not require tissue-specific descriptions. Nevertheless, there are some specificities such as the release of glutamate by brain reactive glial cells (Brown and Bal-Price, 2003; Vesce et al., 2007).The differences may rather reside in the type of insult favouring the increased expression and/or release of a specific class of inflammatory mediators, as well the time after the insult reflecting different stages of the inflammatory process. For these reasons, the analyses of the changes of a battery of inflammatory mediators rather than of a single one is a more adequate measurement of this KE.

Regulatory examples using the KE

CD54 and CD 86 as well as IL-8 expression is used to assess skin sensitization potential (OECD TG 442E). IL-2 expression is used to assess immunotoxicity (and will become an OECD test guideline); for the latter see also doi: 10.1007/s00204-018-2199-7.

LIVER:

When activated, resident macrophages (Kupffer cells) release inflammatory mediators including cytokines, chemokines, lysosomal, and proteolytic enzymes and are a main source of TGF-β1 - the most potent pro-fibrogenic cytokine. Following the role of TGF-β is described in more detail.

Transforming growth factor β (TGF-β) is a pleiotropic cytokine with potent regulatory and

inflammatory activity [Sanjabi et al., 2009; Li and Flavell, 2008a;2008b]. The multi-faceted effects of TGF-β on numerous immune functions are cellular and environmental context dependent [Li et al., 2006]. TGF-β binds to TGF-β receptor II (TGF-βRII) triggering the kinase activity of the cytoplasmic domain that in turn activates TGF-βRI. The activated receptor complex leads to nuclear translocation of Smad molecules,

and transcription of target genes [Li et al., 2006a]. The role of TGF-β as an immune modulator of T cell activity is best exemplified by the similarities between TGF-β1 knockout and T cell specific

TGF-β receptor II knockout mice [Li et al., 2006b; Marie et al., 2006;Shull et al., 1992]. The animals in both of these models develop severe multi-organ autoimmunity and succumb to death within a few weeks after birth [Li et al., 2006b; Marie et al., 2006; Shull et al., 1992]. In addition, in mice where TGF-β signaling is blocked specifically in T cells, the development of natural killer T (NKT) cells, natural regulatory T (nTreg) cells, and CD8+ T cells was shown to be dependent on TGF-β signaling in the thymus [Li et al., 2006b; Marie et al., 2006].

TGF-β plays a major role under inflammatory conditions. TGF-β in the presence of IL-6 drives the differentiation of T helper 17 (Th17) cells, which can promote further inflammation and augment autoimmune conditions [Korn et al., 2009]. TGF-β orchestrates the differentiation of both Treg and Th17 cells in a concentration-dependent manner [Korn et al., 2008]. In addition, TGF-β in combination with IL-4, promotes the differentiation of IL-9- and IL-10-producing T cells, which lack

suppressive function and also promote tissue inflammation [Dardalhon et al., 2008; Veldhoen et al., 2008]. The biological effects of TGF-β under inflammatory conditions on effector and memory CD8+ T cells are much less understood. In a recent study, it was shown that TGF-β has a drastically opposing role on naïve compared to antigen-experienced/memory CD8+ T cells [Filippi et al., 2008]. When cultured *in vitro*, TGF-β suppressed naïve CD8+ T cell activation and IFN-γ production, whereas TGF-β enhanced survival of memory CD8+ T cells and increased the production of IL-17 and IFN-γ [Filippi et al., 2008]. TGF-β also plays an important role in suppressing the cells of the innate immune system.

The transforming growth factor beta (TGF-β) family of cytokines are ubiquitous, multifunctional, and essential to survival. They play important roles in growth and development, inflammation and repair, and host immunity. The mammalian TGF-β isoforms (TGF-β1, β2 and β3) are secreted as latent precursors and have multiple cell surface receptors of which at least two mediate signal transduction. Autocrine and paracrine effects of TGF-βs can be modified by extracellular matrix, neighbouring cells and other cytokines. The vital role of the TGF-β family is illustrated by the fact that approximately 50% of TGF-1 gene knockout mice die in utero and the remainder succumb to uncontrolled inflammation after birth. The role of TGF-β in homeostatic and pathogenic processes suggests numerous applications in the diagnosis and treatment of various diseases characterised by inflammation and fibrosis. [Clark and Coker, 1998; Santibañez et al., 2011; Pohlers et al., 2009] Abnormal TGF-β regulation and function are implicated in a growing number of fibrotic and inflammatory pathologies, including pulmonary fibrosis, liver cirrhosis, glomerulonephritis and diabetic nephropathy, congestive heart failure, rheumatoid arthritis, Marfan syndrome, hypertrophic scars, systemic sclerosis, myocarditis, and Crohn's disease. [Gordon and Globe,2008] TGF-β1 is a polypeptide member of the TGF-β superfamily of cytokines. TGF-β is synthesized as a non-active pro-form, forms a complex with two latent associated proteins latency-associated protein (LAP) and latent TGF- β binding protein (LTBP) and undergoes protolithic cleavage by the endopeptidase furin to generate the mature TGF-β dimer. Among the TGF-βs, six distinct isoforms have been discovered although only the TGF-β1, TGF-β2 and TGF-β3 isoforms are expressed in mammals, and their human genes are located on chromosomes 19q13, 1q41 and 14q24, respectively. Out of the three TGF-β isoforms (β1, β2 and β3) only TGF-β1 was linked to fibrogenesis and is the most potent fibrogenic factor for hepatic stellate cells. [Roberts, 1998; Govinden and Bhoola, 2003]. During fibrogenesis, tissue and blood levels of active TGF-β are elevated and overexpression of TGF-β1 in transgenic mice can induce fibrosis. Additionally, experimental fibrosis can be inhibited by anti-TGF-β treatments with neutralizing antibodies or soluble TGF-β receptors [Qi et al.; 1999; Shek and Benyon , 2004; De Gouville et al., 2005; Chen et al., 2009]. TGF-β1 induces its own mRNA to sustain high levels in local sites of injury. The effects of TGF-β1 are classically mediated by intracellular signalling via Smad proteins. Smads 2 and 3 are stimulatory whereas Smad 7 is inhibitory. [Parsons et al., 2013; Friedman, 2008; Kubiczkova et al., 2012] Smad1/5/8, MAP kinase (mitogen-activated protein) and PI3 kinase are further signalling pathways in different cell types for TGF-β1 effects.

TGF-β is found in all tissues, but is particularly abundant in bone, lung, kidney and placental tissue. TGF-β is produced by many, but not all parenchymal cell types, and is also produced or released by infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets. Following wounding or inflammation, all these cells are potential sources of TGF-β. In general, the release and activation of TGF-β stimulates the production of various extracellular matrix proteins and inhibits the degradation of these matrix proteins. [Branton and Kopp, 1999]

TGF-β 1 is produced by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells, and its expression serves in both autocrine and paracrine modes to control the differentiation, proliferation, and state of activation of these immune cells. [Letterio and Roberts; 1998]

In the liver TGF-β1 is released by activated Kupffer cells, liver sinusoidal endothelial cells, and platelets; in the further course of events also activated hepatic stellate cells express TGF-β1. Hepatocytes do not produce TGF-β1 but are implicated in intracellular activation of latent TGF-β1. [Roth et al., 1998; Kisseleva and Brenner, 2007; Kisseleva and Brenner, 2008; Poli, 2000; Liu et al., 2006]

TGF-β1 is the most established mediator and regulator of epithelial-mesenchymal-transition (EMT) which further contributes to the production of extracellular matrix. It has been shown that TGF-β1 mediates EMT by inducing snail-1 transcription factor and tyrosine phosphorylation of Smad2/3 with subsequent recruitment of Smad4. [Kolios et al., 2006; Bataller and Brenner, 2005; Guo and Friedman,2007; Brenner,2009; Kaimori et al., 2007; Gressner et al., 2002; Kershenobich Stalnikowitz and Weisssbrod, 2003; Li et al., 2008; Matsuoka and Tsukamoto, 1990; Kisseleva and Brenner, 2008; Poli, 200; Parsons et al., 2007; Friedman 2008; Liu et al., 2006]

TGF-β1 induces apoptosis and angiogenesis in vitro and in vivo through the activation of vascular endothelial growth factor (VEGF) High levels of VEGF and TGF-β1 are present in many tumors. Crosstalk between the signalling pathways activated by these growth factors controls endothelial cell apoptosis and angiogenesis. [Clark and Coker; 1998]

How it is Measured or Detected

The specific type of measurement(s) might vary with tissue, environment and context and will need to be described for different tissue contexts as used within different AOP descriptions.

In general, quantification of inflammatory markers can be done by:

- qRT-PCR (mRNA expression)
- ELISA
- Immunocytochemistry
- Immunoblotting

For descriptions of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014

LIVER:

There are several assays for TGB-β1 measurement available.

e.g. Human TGF-β1 ELISA Kit. The Human TGF-β 1 ELISA (Enzyme –Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TGF-β1 in serum, plasma, cell culture supernatants, and urine. This assay employs an antibody specific for human TGF-β1 coated on a 96-well plate. Standards and samples are pipetted into the wells and TGF-β1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TGF-β1 antibody is added. After washing away unbound biotinylated antibody, HRP- conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of TGF-β1 bound. The StopSolution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm [Mazzieri et al., 2000]

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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

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:442 - Inhibition of voltage gate sodium channels leading to impairment in learning and memory during development	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

Life Stage Applicability

Life Stage	Evidence

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).

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, 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 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).

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).

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).

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: 1765: Binding, SH/SeH proteins involved in protection against oxidative stress leads to Protection against oxidative stress, decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	
Female	

Experimental evidences has been observed in rat, mice and human cells (Agrawal, 2015; Meinerz, 2011; Branco, 2017)

Key Event Relationship Description

Thiol (SH) and selenol (SeH) compounds exhibit reactivity toward electrophiles and oxidants and have high binding affinities for metals (Higdon, 2012; Nagy, 2013; Winterbourn, 2008; Winther, 2014). Glutathione is a thiol-containing tripeptide acting as a cofactor for the enzyme peroxidase and thus serving as an indirect antioxidant donating the electrons necessary for its decomposition of H_2O_2, and is also involved in many other cellular functions (Kohen, 2002). Selenoproteins contain selenocysteine amino acid residues. The selenoprotein family is composed of proteins exerting diverse functions, among them several are oxidoreductases classified as antioxidant enzymes (Labunskyy, 2014; Reeves, 2009). Relevant for this KER there are two well-studied selenoprotein families which are described to be expressed in the brain; (i) the Glutathione Peroxidase (GPx) family, involved in detoxification of hydroperoxides; (ii) the Thioredoxin Reductase (TrxR) family, involved in the regeneration of reduced thioredoxin (Pillai, 2014), but also the less studied SelH, K, S, R, W, and P selenoproteins (Pisoschi, 2015; Reeves, 2009).

As summarized in the table 1, binding to the thiol/selenol groups of the selenoproteins cited above can result in structural modifications of these proteins, which in turn inhibits their catalytic activity and thereby reduces or blocks their metabolic capacity to neutralize reactive oxygen species (Fernandes, 1996; Rajanna, 1995). Similarly, binding to the thiol group of glutathione will decrease its anti-oxidant capacity.

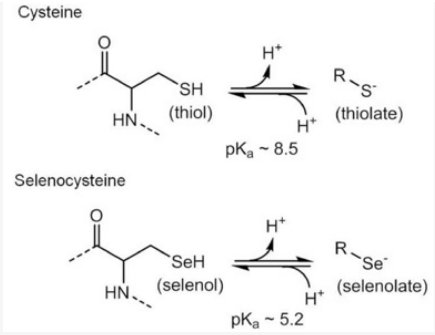

Figure (Poole, 2015) Structures of cysteinyl and selenocysteinyl residues within proteins. The aminoacyl groups are shown to the left, with dotted lines representing peptide bonds to the next residue on either side. Both protonated (left) and deprotonated (right) forms of these amino acids are depicted with average pKa values.

Evidence Supporting this KER

Biological Plausibility

GPx family

GPxs are tetrameric enzymes. Their thiol groups can either act directly as a reductant, or catalyze reduction of hydrogen peroxide and/or phospholipid hydroperoxides through glutathione co-factors (Hanschmann, 2013, Labunskyy, 2014).

TrxR family

TxRs are homodimeric flavoenzymes, which mediate the reduction of oxidized Txn at the expense of NADPH (Birben, 2012). Inhibition of TrxR enzymes has been shown to lead to oxidative stress (Branco, 2017).

SelP

Downregulation of intracellular SelP by use of small interfering RNA (siRNA) impaired the viability of human astrocytes and made them more susceptible to hydroperoxide-induced oxidative stress, pointing to a direct contribution of SeP to ROS clearance (Steinbrenner, 2006).

Table 1

Selenoprotein family	Protein name	Normal brain function	Disruption leading to oxidative stress	Reference
Glutathione	GSH	GSH is a major endogenous antioxidant functioning directly in neutralization of free radicals and reactive oxygen compounds. GSH is the reduced form of glutathione and its SH group of cysteine is able to reduce and/or maintain reduced form of other molecules.	Disruptions leads to increased oxidative stress and apoptosis.	Hall, 1999 Dringen, 2000
Glutathione Peroxidase (GPx) Family	GPx1	Peroxide/ROS reduction (Promotes neuroprotection in response to oxidative challenge). Brain expression levels are highest in microglia and lower levels detected in neurons.	Brains of GPx1–/– mice are more vulnerable to mitochondrial toxin treatment, ischemia/ reperfusion, and cold-induced brain injury. Cultured neurons from GPx1–/– mice were reported to be more susceptible to Aβ-induced oxidative stress, and addition of ebselen reversed this.	Lindenau, 1998 Klivenyi, 2000 Flentjar, 2002 Crack, 2001 and 2006
	GPx4	Reduction of phospholipid Hydroperoxides. Only in neurons during normal conditions.	Brains of GPx4+/– mice were shown to have increased lipid peroxidation (a sign of oxidative stress). Injury-induced GPx4 expression in astrocytes. In vivo over expression of GPx4	Ran, 2004 Borchert, 2006 Savaskan, 2007 Chen, 2008

			protects against oxidative stress-induced apoptosis.	
Thioredoxin Reductase (TrxR) Family	TrxR1 TrxR2	Cytocsolic, mitochondrial, nuclear localization. Contribute to the reduction of hydrogen peroxide and oxidative stress, and regulates redox-sensitive transcription factors that control cellular transcription mechanisms. Regulate the induction of the antioxidant enzyme heme oxygenase 1 (HO-1).	Overexpression of human Trx1 and Trx2 protects retinal ganglion cells against oxidative stress-induced neurodegeneration. Exogenously administered human rTrx ameliorates neuronal damage after transient middle cerebral artery occlusion in mice, reduces oxidative/nitrative stress and neuronal apoptosis after cerebral ischemia/reperfusion injury in mice	Gladyshev, 1996 Zhong, 2000 Hattori, 2004 Trigona, 2006 Papp, 2007 Munemasa, 2008 Arbogast, 2010 Ma, 2012 Burk, 2013 Pitts, 2014
Other relevant seleno-proteins	SelH	Nuclear localization. Redox sensing.	Hypersensitivity of SelH shRNA HeLa cells to paraquat- and H2O2-induced oxidative stress.	(Panee, 2007) (Novoselov, 2007) (Wu, 2014)
	SelK	Transmembrane protein localized to the ER membrane. ER homeostasis and oxidative stress response.	Protects HepG2 cells from ER stress agent-induced apoptosis. Overexpression of SelK attenuated the intracellular reactive oxygen species level and protected cells from oxidative stress-induced toxicity in cardiomyocytes	(Shchedrina, 2011) (Du, 2010) (Lu, 2006)
	SelS	Transmembrane protein localized to the ER membrane. Catalyze the reduction of disulfide bonds and peroxides.	SelS overexpression increased astrocyte resistance to ER-stress and inflammatory stimuli, and suppression of SelS compromised astrocyte viability.	(Liu, 2013) (Fradejas, 2011) (Fradejas, 2008) (Gao, 2007)
	MSRB1, SelR, SelX	Function in reduction of oxidized methionine residues, and actin polymerization.	Induce expression of MSRB1 protects neurons from amyloid β-protein insults in vitro and in vivo.	(Lee, 2013) (Moskovitz, 2011)(Pillai, 2014)
	SelW	Expressed in synapses. Plays an antioxidant role in cells.	Rat in vivo overexpression of SelW was shown to protect glial cells against oxidative stress caused by heavy metals and 2,20-Azobis. Silencing of SelW made neurons more sensitive to oxidative stress.	(Reeves, 2009) (Sun, 2001) (Loflin, 2006) (Raman, 2013) (Chung, 2009)
	SelP	Is important for selenium transport, distribution and retention within the brain. Acts as a ROS-detoxifying enzyme.	SelP-/- mice show neurological dysfunction and that Se content and GPx activity were reduced within brain, Se supplementation to diet attenuated. neurological dysfunctions. SelP-/- mice have reported deficits in PV-interneurons due to diminished antioxidant defense capabilities. Decreased neuronal selenoprotein	(Steinbrenner, 2009)(Arbogast, 2010)(Zhang, 2008) (Hill, 2003;Hill, 2004) (Cabungcal, 2006) (Pitts, 2012) (Byrns, 2014)

		Protects human astrocytes from induced oxidative.	synthesis may be a functional outcome of SelP Colocalization of Sel P with amyloid plaques SelP can function as an antioxidant enzyme against reactive lipid intermediates	 (Schomburg, 2003) (Rock, 2010)

Empirical Evidence

Mercury

Thiol- and selenol containing proteins have a high affinity for binding metals which contributes to the target site – brain – distribution of such toxicants (Farina, 2011).

The selenol group (-SeH) of selenocysteines is generally more reactive than thiols (-SH) towards mercury (Sugiura 1976, Khan, 2009). Methyl mercury (MeHg) can target both the GPx and TrxR proteins thereby decreasing protection against oxidative stress and therefore causing increased oxidative stress and neurotoxicity (Branco, 2017, Carvalho, 2008, Farina, 2011).

Note: The binding of $HgCl_2$ and MeHg is always studied in vitro on the isolated protein, whereas the effects on the activity of the proteins involved in protection against oxidative stress is mostly studied in isolated cells, mitochondrial fractions or in animals. Therefore the concentrations cannot be compared. Binding of Hg to thiol groups and to various selenium-containing proteins: Glutathione, thioredoxin reductase, thioredoxin, glutaredoxin, glutathione reductase was measured using purified proteins (Carvahlo et al., 2008, 2011; Wiederhold et al., 2010; Sugiura et al., 1978; Arnold et al., 1986; Han et al., 2001; Qiao et al., 2017).

Table 2

KE_{up} **Binding, Thiol/seleno-proteins involved in protection against oxidative stress**	**KE_{down}** **Decreased protection against oxidative stress**	**Species; in vivo / in vitro**	**Stressor**	**Dose/ conc. +** **Duration of exp.**	**Protective/ aggravating evidence**	**Reference**
Binding of 2.5 mol of Hg^{2+} /mol of TrX1 (Carvahlo et al., 2008)	Inhibition of TrX Inhibition of TrXR	Recombinant rat TrX HeLa and HEK293 cells	$HgCl_2$	IC_{50} 7.2 nM	Selenite (5 mM)	(Carvahlo et al., 2008, 2011)
Binding of 5 mol of Hg^{2+} /mol of TrX1 (Carvahlo et al., 2008)	Inhibition of TrX Inhibition of TrXR	Recombinant rat TrX HeLa and HEK293 cells	MeHg	IC_{50} 19.7 nM	Selenite (5 mM)	(Carvahlo et al., 2008, 2011)
Binding to GR and GrX (Carvahlo et al., 2008)	Total inhibition	Purified proteins	Hg2+	10 nM		(Carvahlo et al., 2008)
Binding to GR and GrX (Carvahlo et al., 2008)	50% of inhibition	Purified proteins	MeHg	80 nM		(Carvahlo et al. 2008)
	Inhibition of TrxR and GSH activities. TrxR activity – *cytosolic: 0.7 fold; mitochondrial: 0.4 fold)* *TrxR1&2 expression – slight decrease, not quantified* *GSH – 0.7-*	Human neuroblastoma cells (SH-SY5Y)	MeHg	1 µM		(Branco, 2017)

	fold Depletion of GSH levels. *GSH-activity:* *10µM – 0.75-fold* *30µM – 0.6-fold* *100µM – 0,5-fold*	Mouse brain mito-chondrial-enriched fractions	MeHg	10, 30, and 100 µM 30 minutes	The co-incubation with diphenyl diselenide (100 µM)completely prevented the disruption of mitochondrial activity.	(Meinerz, 2011)
	Depleted GSH levels.	Adult male Wistar rats	mercuricchloride	30ppm in drinking water		(Agrawal, 2015)

Acrylamide (acrylamide is a common food contaminant generated by heat processing)

No literature supporting the link "SH/SeH binding leads to decreased protection against oxidative stress" for ***acrylamide as stressor*** *in brain/neural tissue can be found.*

Uncertainties and Inconsistencies

Another important group of thiol-containing proteins are the metal-binging detoxifying metallothioneins. This protein family bind mercury and lead, and this binding thus serves as a protective mechanism and also protects against metal toxicity and oxidative stress (Aschner, 2006).

Lactational exposure to methylmercury (10 mg/L in drinking water) significantly increased cerebellar GSH level and GR activity. Possibly a compensatory response to mercury-induced oxidative stress (Franco, 2006).

MeHg was shown to inhibit cerebral thioredoxin reductase activity in vitro but not in brain of mice (Wagner et al., 2010). However, it has to be noted that the exposure of mice to MeHg was only 24h.

Inhibition og GR and GrX by Hg2+ and MeHg was observed on the puried protein, but not in HeLa cells incubated with the same concentrations for 24h (Carvahlo et al., 2008).

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Relationship: 1766: Protection against oxidative stress, decreased leads to Oxidative Stress

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

The link between decrease in antioxidant protection and induction of oxidative stress can be found in Zebrafish, rodents (mouse and rat) and in man, but may not be restricted to these species.

Key Event Relationship Description

High levels of oxidizing free radicals can be very damaging to cells and molecules within the cell. As a result, the cell has important defense mechanisms to protect itself from ROS, including reducing agents, glutathione peroxidases, thioredoxin reductases. Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses. Ensuing from this definition, a decrease in cellular antioxidant protection will lead to the increase of oxidative stress.

Evidence Supporting this KER

Biological Plausibility

The cell has important defense mechanisms to protect itself from oxidative stress. The cellular defense mechanisms are numerous and include repair mechanisms, prevention mechanisms, physical defenses, as well as antioxidant defense such as antioxidant enzymes, low-molecular-weight antioxidants and chelating agents (Kohen, 2002). Whenever one or many of these mechanisms are decreased, the balance will tilt towards the production of ROS, and thus generate oxidative stress. In this KER we focus on the decreased protection due to interference with the antioxidant defense system.

Empirical Evidence

KE_{up} **Decreased protection against oxidative stress**	**KE_{down}** **Oxidative stress**	**species; in vivo / in vitro**	**Stressor**	**Dose/ conc. +** **Duration of exp.**	**Protective/ aggravating evidence**	**Reference**
Post-transcriptional effects on GPx1 and TrxR1 expression and activity *TrxR1 – 2-fold* *GPx1 – 0.6-fold*	Disturbance of redox-response and induction of oxidative stress *SOD – 2-fold* *ROS – increased*	Mouse myoblast C2C12,	MeHg	0.4 µM 9 h	Treatment with ebselen suppressed MeHg-induced oxidative stress	(Usuki, 2011)
Inhibition of TrxR and GSH activities	Oxidative stress shown by shift in GSSG/GSH ratio	Human neuroblastoma cells (SH-	MeHg	1 µM	Se supplementation gave some extent of	(Branco, 2017)

TrxR1&2 – 0.6-fold *GSH – 0.7-fold*	*GSSG/GSH – 1.5-fold*	SY5Y)			oxidative stress protection	
Inhibition of GPx activity *GPx – 0.4-fold*	Increased ROS formation and lipid peroxidation *ROS – 1.75-fold* *Total peroxidase – 4.5-fold* *Lipid perox. – 3-fold*	Mouse brain	MeHg	40 mg/L in drinking water 21-days	Incubation of mitochondrial-enriched fractions with exogenous GPx completely blocked MeHg-induced mitochondrial lipid peroxidation	(Franco, 2009)
Inhibition of GPx activity *GPx – 0.7-fold*	Increased ROS formation and lipid peroxidation *Total H_2O_2 – 1.5-fold*	Human neuro-blastoma SH-SY5Y cells.	MeHg	1 µM (nominal)	Inhibition of GPx substantially enhanced MeHg toxicity	(Franco, 2009)
Decreased GPx1, activity in cerebral cortex and hippocampus *GPx1 – 0.5-fold*	Induction of oxidative stress (oxidative damage product from the reaction of ROS and deoxy-thymidine in DNA)	Male C57BL/6NJcl mice	MeHg	1.5 mg kg^{-1} day^{-1} 6-weeks		(Fujimura, 2017)
Depletion of GSH levels *GSH-activity:* *10µM – 0.75-fold* *30µM – 0.6-fold* *100µM – 0,5-fold*	Increased glutathione oxidation, hydroperoxide formation (xylenol orange assay) and lipid peroxidation end-products (thiobarbituric acid reactive substances, TBARS). *Mitochondrial viability*: *10µM – 0.75-fold* *30µM – 0.6-fold* *100µM – 0,5-fold* *Total hydroperoxidases:* *10µM – 1.0-fold* *30µM – 1.2-fold* *100µM – 1.75-fold*	Mouse brain mitochondrial-enriched fractions	MeHg	10, 30, and 100 µM 30 minutes	The co-incubation with diphenyl diselenide (100 µM) completely prevented the disruption of mitochondrial activity as well as the increase in TBARS levels. thiol peroxidase activity of organoselenium compounds accounts for their protective actions against methylmercury-induced oxidative stress	(Meinerz, 2011)
Depletion of mono- and disulfide glutathione in neuronal, glial and mixed cultures *GSH activity – 0.83-fold*	increased reactive oxygen species (ROS) formation measured by dichlorodihydro-fluorescein (DCF) fluorescence *DCF – 1.2-1.5-fold*	Mouse primary cortical cultures	MeHg	5 µM 24h	glutathione monoethyl ester (GSHME : 100 µM) protects against oxidative stress formation	(Rush, 2012)
Reduced glutathione (GSH) content	Increased lipid peroxidation and	Adult male albino	Dimethylmercury	10 mg/kg bw	Supplementation with Se (2 mmol/kg and 0.5 mg/kg partially	(Deepmala,

decreased in liver, kidney and brain.	generation of reactive oxygen species	Sprague-Dawley rat	(DMM)	3-days	protected against DMM-induced tissue damage.	2013)
Reduced glutathione (GSH) level and acetyl cholinesterase activity, as well as reduced antioxidant enzyme glutathione peroxidase (GPx)	Increased lipid peroxidation level and DNA damage.	Adult male Sprague-Dawley rats	MeHg	1 mg kg^{-1} orally 6 months		(Joshi, 2014)
Depleted GSH levels	Antioxidant imbalance and lipid peroxidation.	Adult male Wistar rats	mercuricchloride	30 ppm in drinking water		(Agrawal, 2015)
GPx1 significantly decreased prior to neurotoxic effects being visible *GPx1 – 0.7-fold*	Increased lipid peroxidation and later neuronal cell death. *Lipid peroxidation - 1.75-fold*	Primary cultured mouse cerebellar granule cells	MeHg	300 nM nominal 24h	Overexpression of GPx-1 prevented MeHg-induced neuronal death	(Farina, 2009)
Reduction of GPx activity and increased glutathione reductase activity *GPx – 0.7-fold*	Increased oxidative stress – shown by increased TBA-RS and 8-OHdG content, as well as reduction of complexes I, II, and IV activities *H_2O_2 – 1.6-fold*	Adult male Swiss albino mice	MeHg	3–5 µg/g brain tissue 21-days	Treatment with diphenyl diselenide $(PhSe)_2$ (5 µmol/kg) reversed MeHg's inhibitory effect on mitochondrial activities, as well as the increased oxidative stress parameters	(Glaser, 2013)
Decreased level of GSH in blood, liver, heart, brain, lung and testis *GSH – 0.4-0.7 fold*	Lipid peroxidation (increase in malondialdehyde levels in blood, liver, heart, brain, lung and testis) *Lipid peroxidation – 1.4-2.0 fold*	Rats	Acrylamide	15 mg kg^{-1} day^{-1} 60 days gastric gavage	All effects prevented by co-treatment with boron	(Acaroz, 2018)
Decreased level of GSH in liver, kidney, brain, lung and testis *GSH – 0.4-0.6 fold*	Lipid peroxidation (increase in malondialdehyde levels liver, kidney, brain, lung and testis) *Lipid peroxidation – 1.6-2.0 fold*	Rats	Acrylamide	40 mg kg^{-1} day^{-1} 10 days i.p.	All effects prevented by co-treatment with resveratrol	(Alturfan, 2012)
Decreased level of GSH and decreased activity of GPx and SOD in cerebellum *GSH – 0.5 fold* *GPx – 0.6 fold*	Increased lipid peroxidation (MDA) and DNA fragmentation (comet assay) in cerebellum *Lipid peroxidation –1.9 fold*	Rats	Acrylamide	40 mg kg^{-1} day^{-1} 12 days gavage	All effects prevented by melatonin	Pan et al., 2015

SOD – 0.7 fold						
Decreased level of GSH/GSSG ratio *GSH/GSSG ratio – 0.85 to 0.70 after 0.5-1 mM, 24h and 0.85 to 0.45 fold after 01-1 mM, 96h*	Increased ROS production, increased lipid peroxidation (4-HNE), increased oxidative DNA damage (8-OHdG) *ROS – 1.5 to 3.2 fold after after 0.5-1 mM, 24h* *4-HNE: 1.6 fold after 1mM, 96h* *8-OHdG: 1.7 and 2.1 fold after 0.1 and 1 mM, 96h*	Primary cultured mouse astrocytes and microglia	Acrylamide	0-1 mM 24-96 h		Zhao et al., 2017

Uncertainties and Inconsistencies

No uncertainties, since a decrease in protection against oxidative stress leads, by definition, to an increase in oxidative stress

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Relationship: 1685: Oxidative Stress leads to Glutamate dyshomeostasis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Male	High
Female	High

Experimental evidences has been observed mainly in rodent, but due to occurrence of oxidative stress and the presence of glutamate in different taxa, it may be much broader, as suggested by similar observations in C. elegans (Wu et al., 2015).

Key Event Relationship Description

In the central nervous system (CNS), glutamate (Glu) is rapidly taken up at the synaptic cleft to mitigate potential excitotoxicity (Meldrum, 2000). Reuptake is carried out by the electrochemical gradient of Glu across the plasma membrane and is accomplished by Glu transporter proteins, referred to as excitatory amino acid transporters (EAATs). These transporter proteins are predominantly expressed in astrocytes, but they are also be found in other neural cells, such as oligodendrocyte, neuron, and microglia membranes (Danbolt, 2001). Functional Glu transporters are located on cell surface membranes. The activities of these transporters are regulated by a redistribution of these proteins to or from the plasma membrane (Robinson 2002), under the control of several signaling pathways. Five different families of EAATs have been recognized (EAAT1–EAAT5). They vary in Na^+ and/or K^+ coupling abilities. Their names differ based on the presence of the transporter in human or in other mammals (see Table 1).

Transporter (Human)	Transporter (Mammals)	Occurrence (Cell)
EAAT1	GLAST	Astrocyte, oligodendrocyte, microglia
EAAT2	GLT-1	Astrocyte, oligodendrocyte
EAAT3	EAAC1	Neuron (somatodendritic), astrocyte (low)
EAAT4	EAAT4	Purkinje cell, astrocyte
EAAT5	EAAT5	Müller cell (retina)

Table 1: Glu transporters in human and mammals and their occurrence in CNS cells. From Rajda et al., 2017

These transporters co-localize with, form physical (co-immunoprecipitable) interactions with, and functionally couple to various 'energy-generating' systems, including the Na(+)/K(+)-ATPase, the Na^+/Ca^{2+} exchanger, glycogen metabolizing enzymes, glycolytic enzymes, and mitochondria/mitochondrial proteins. This functional coupling is bi-directional with many of these systems both being regulated by glutamate transport and providing the 'fuel' to support glutamate uptake (Robinson and Jackson, 2016). The Na^+ gradient, which depends on Na/K ATPase pump and consequently of ATP production and intracellular levels, provides the energy to move Glu from the outside into the cells, accompanied by two Na^+ and an H^+ ; at the same time, K^+ moves in the opposite direction (Boron and Boulpaep, 2003). Mitochondrial dysfunction leads to a decrease in ATP synthesis, impaired Ca^{2+} content, and concomitant increase in the levels of ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) (Beal, 2005). Free radicals, which are electrically unstable, have a central role in several physiological and pathological processes. Both ROS and RNS originate from endogenous and exogenous sources. Mitochondria, endoplasmic reticulum, peroxisomes, phagocytic cells, and others serve as endogenous sources, and environmental factors, such as alcohol, tobacco, pollution, industrial solvents, pesticides, heavy metals, specified medicines, etc. make up the prepondarance of exogenous factors. Significant amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS,) are formed during oxidative phosphorylation, when the greatest amount of ATP is produced. Cellular antioxidants production serves as a countermeasure against this process (Su et al., 2013; Szalardi et al., 2015). Most cells, including astrocytes, have protective mechanisms against ROS, predominantly in the form of the tripeptide thiol, glutathione (GSH) (Hsie et al., 1996). This process stays in a highly sensitive balance. In the specific case when ROS and RNS synthesis exceeds antioxidant synthesis it results in oxidative stress (Reddy, 2006; Ghafouribar et al., 2008; Su et al., 2013; Szalardi et al., 2015; Valko et al., 2007; Yankovskaya et al., 2003; Senoo-Matsuda et al., 2003; Schon and Manfredi, 2003).

Evidence Supporting this KER

Biological Plausibility

Due to the tight coupling of Glu transporters with energy production, and to the important role of Glu transporters in Glu homeostasis, perturbations of energy metabolism such as mitochondrial dysfunction and increased production of ROS lead to Glu dyshomeostasis (Boron and Boulpaep, 2003). In particular, it was shown that ROS inhibit glutamate uptake by astrocytes (Sorg et al., 1997), and that glutamate release is mediated by ROS-activated volume-sensitive outwardly rectifying anion channels (Liu et al., 2009).

Empirical Evidence

Porciuncula et al., (2003). Methylmercury (2-10 µM) inhibits glutamate uptake in synaptic vesicles isolated from rat brain in a concentration-dependent manner (with LD_{50} at 50 µM). It also inhibits the H^+-ATPase activity in a concentration-dependent manner with similar LD_{50}. This suggests that the vesicular glutamate uptake is impaired by methylmercury and that this effect involves the H^+-ATPase.

Roos et al., (2009). Methylmercury induced ROS production in rat brain cortical slices after 2h exposure at 100 and 200 µM and after 5h exposure at 50 µM. Guanosine (0.5 - 5 µM), ebselen (1-5 µM) and diphenyl diselenide (1-5 µM) blocked the methylmercury-induced ROS production. The inhibitor of NMDA receptors, MK801 (50 µM) equally blocked the methylmercury-induced ROS production by two potential mechanisms of action: (i) mercury by affecting mitochondria iincreased ROS formation, which decrease glutamate uptake and consequently increased extracellular glutamate acting on NMDA receptors; (ii) The ROS formation is secondary to overstimulation of NMDA receptors, due to mercury-induced decrease in glutamate uptake.

Roos et al., (2011). Experiments performed in isolated mitochondria from rat liver slices showed that methylmercury (25 µM) increased ROS production (measured by dichlorofluoroscein). Methionine treatment (50-250 µM) was effective in reducing ROS formation.

Juarez et al., (2002) Microdialysis probe in adult Wistar rats showed that acute exposure to methylmercury (10, 100 µM) induced an increase release of extracellular glutamate (9.8 fold at 10 µM and 2.4 fold at 100 µM). This extracellular glutamate level remained elevated at least 90 min following methylmercury exposure.

Allen et al., (2001). Cerebral cortical astrocytes were treated with methylmercury (1 µM for 24h or 10 µM for 30 min) and loaded with [U-^{13}C] glutamate. In the methylmercruy-treated group, a decrease of [U-^{13}C] lactate was observed. This lactate can only be derived from mitochondrial metabolism, via the tricarboxylic acid, showing a link between mitochondrial dysfunction and glutamate metabolism. In addition, the decreased lactate production might be detrimental to surrounding cells, since

lactate has been shown to be an important substrate for neurons.

Uncertainties and Inconsistencies

The relationship between oxidative stress associated to mitochondrial dysfunction and glutamate dyshomeostasis is complex and may be bidirectional. Glutamate dysfunction, due to decreased glutamate uptake, can secondarly induce increased ROS production and consequently oxidative stress.

The astrocytic enzyme glutamine synthetase (GS), transforming glutamate in glutamine, which is taken up by neurons, is also a SH-containing protein, which is inhibited by mercury binding (Kwon and Park, 2003). This participate to glutamate dyshomeostasis linking this KE directly to the MIE.

Quantitative Understanding of the Linkage

According to **Porciuncula et al., (2003),** a decrease of 50% of H^+-ATP activity was associated to a decrease of 50% of glutamate uptake following exposure of synaptic vesicles with 5 uM of methylmercury.

Xu et al. (2012) and **Feng et al. (2014)** observed that in rats treated with 12 umoles/kg for 4 weeks a 4-fold increase in ROS level in cerebral cortex, and a 2-fold increase in protein and DNA peroxidation were associated with about 20% increase of glutamate and 30% decrease of glutamine.

Known Feedforward/Feedback loops influencing this KER

In case of glutamate dyshomeostasis, when extracellular concentrations are very high (5 – 10 mM), a mechanism of toxicity called oxidative glutamate toxicity can be observed. It is mediated by an inhibition of cystein uptake leading to a depletion of GSH (Kritis et al., 2015). The GSH depletion decreases the protection against oxidative stress and exacerbates oxidative stress, which, in turn, exacerbates glutamate dyshomeostasis.

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Relationship: 1686: Glutamate dyshomeostasis leads to Cell injury/death

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Support for the link between glutamate dyshomeostasis and cell injury /death can be found in rats, and mouse. However, as the neurotransmitter glutamate is already found in insects, it is plausible that this KER is valid throughout taxa (Harris et al., 2014).

Key Event Relationship Description

Glutamate is the major excitatory neurotransmitter in the mammalian CNS, where it plays key roles in development, learning, memory and response to injury. However, glutamate at high concentrations at the synaptic cleft acts as a toxin, inducing neuronal injury and death (Meldrum, 2000; Ozawa et al., 1998). Glutamate-mediated neurotoxicity has been dubbed as “excitotoxicity”, referring to the consequence of the overactivation of the *N*-methyl D-aspartate (NMDA)–type glutamate receptors (cf AOP 48), leading to increased Na^+ and Ca^{2+} influx into neurons (Choi, 1992; Pivovarova and Andrews, 2010). Increased intracellular Ca^{2+} levels are associated with the generation of oxidative stress and neurotoxicity (Lafon-Cazal et al., 1993). Accordingly, the control of extracellular levels of glutamate dictates its physiological/pathological actions and this equilibrium is maintained primarily by the action of several glutamate transporters (such as GLAST, GLT1, and EAAC1) located on astrocytic cell membranes, which remove the excitatory neurotransmitter from the synaptic cleft, keeping its extracellular concentrations below toxic levels (Anderson and Swanson, 2000; Maragakis and Rothstein, 2001; Szydlowska and Tymianski, 2010).

In addition to synaptic transmission, physiological stimulation of glutamate receptors can mediate trophic effects and promote neuronal plasticity. During development, NMDA receptors initiate a cascade of signal transduction events and gene expression changes primarily involving Ca^{2+}-mediated signaling, induced by activation of either Ca^{2+}- permeable receptor channels or voltage-sensitive Ca^{2+} channels. The consecutive activation of major protein kinase signaling pathways, such as Ras-MAPK/ERK and PI3-K-Akt, contributes to regulation of gene expression through the activation of key transcription factors, such as CREB, SRF, MEF-2, NF-kappaB. Metabotropic glutamate receptors can also engage these signaling pathways, in part by transactivating receptor tyrosine kinases. Indirect effects of glutamate receptor stimulation are due to the release of neurotrophic factors, such as brain derived neurotrophic factor through glutamate-induced release of trophic factors from glia. The trophic effect of glutamate receptor activation is developmental stage-dependent and may play an important role in determining the selective survival of neurons that made proper connections. During this sensitive developmental period, interference with glutamate receptor function may lead to widespread neuronal loss (Balazs, 2006).

Evidence Supporting this KER

Biological Plausibility

Glutamate dyshomeostasis and in particular excess of glutamate in the synaptic cleft will lead to overactivation of ionotropic glutamate receptors and cause cell injury/death, as described in AOP 48. The excess of glutamate can result from decreased uptake in astrocytes (Aschner et al., 2000; Brookes and Kristt, 1989), or neurons (Moretto et al., 2005; Porciuncula et al., 2003). But also from the increased release (Reynolds and Racz, 1987). This neurotoxic cascade involves calcium overload and ROS production leading to oxidative stress (Ceccatelli et al., 2010; Lafon-Cazal, 1993; Meldrum, 2000; Ozawa, 1998). Chemicals binding to sulfhydryl (SH)-/seleno-proteins cause a direct oxidative stress by perturbing mitochondrial respiratory chain proteins and by decreasing anti-oxidant defense mechanism (see KER : MIE to KEdown oxidative stress) and an indirect oxidative stress via perturbation of glutamate homeostasis/excitotoxicity. Thus, there may be some redundancy in the empirical support between this KER and the KER linking KEup oxidative stress and KEdown cell injury/death.

Glutamate has been shown to regulate BDNF production (Tao et al., 2002). Accordingly, glutamate may also indirectly contribute to cell injury/death by inducing modifications in the brain levels of trophic factors, since it is known that changes in trophic support can lead to cell injury/death, as well as to perturbation in the physiological establishment of neuronal network (Zhao et al., 2017).

Empirical Evidence

KE_{up} Glutamate dyshomeostasis	KE_{down} Cell injury/death	species; developmental stage of exposure to stressor	Stressor	Dose or conc. Duration	Protective/ aggravating evidence	Reference
Dose-dependent increase in glutamate content in cerebral cortex (+ 60% at 12 µmol kg^{-1})	Increased apoptosis rate measured by flow cytometry (+ 1100% at 12 µmol kg^{-1})	Rat adult exposure	MeHgCl	4 µmol kg^{-1} 12 µmol kg^{-1} i.p injection 5 injections per week	Pretreatment with dextro-methorphan (low-affinity, noncompetitive NMDAR antagonist) partially decreased Glu content and apoptosis	(Feng et al., 2014)

				during 4 weeks	induced by MeHgCl	
Dose-dependent increase in glutamate content in cerebral cortex (+ 22% at 12 µmol kg^{-1})	Increased apoptosis rate measured by flow cytometry (+ 850% at 12 µmol kg^{-1})	Rat adult exposure	MeHgCl	4 µmol kg^{-1} 12 µmol kg^{-1} i.p injection 5 injections per week during 4 weeks	Pretreatment with memantine (low-affinity, noncompetitive NMDAR antagonist) partially decreased Glu content and apoptosis induced by MeHgCl	(Liu et al., 2013)
Increase in glutamate content in cerebral cortex (1.12 fold at 12 µmol kg^{-1})	Increased apoptosis rate measured by flow cytometry (+ 630% at 12 µmol kg^{-1})	Rat adult exposure	MeHgCl	4 µmol kg^{-1} 12 µmol kg^{-1} i.p injection 5 injections per week during 4 weeks	Pretreatment with MK801 (noncompetitive NMDAR antagonist) partially decreased apoptosis induced by MeHgCl	(Xu et al., 2012)
Decreased glutamine uptake	Reduction in inner mitochondrial membrane potential	Rat astrocyte cultures	MeHg	1, 5, 10 µM 1 and 5 min		(Yin et al., 2007)
Changes in intracellular glutamate concentration	Cell death measured by MTT reduction and LDH release	Mouse astrocytes, neurons in mono- or co-cultures	MeHg	1-50 µM 24h		(Morken et al., 2005)
Concentration-dependent inhibition of glutamate uptake and stimulation of glutamate release	Cell death measured by MTT reduction	Mouse cerebellar granule cells in culture	$HgCl_2$ MeHgCl	10^{-7}-10^{-4} M 10 min		(Fonfria et al., 2005)
Dose-dependent decreased cortical glutamate concentration	Dose-dependent abnormal neuronal morphology	Rat Young (3-4 weeks)	Acrylamide	5, 15, 30 mg kg^{-1} 5 injections per week during 4 weeks gavage		(Tian et al., 2015)

Uncertainties and Inconsistencies

No uncertainty or inconsistency reported yet.

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Relationship: 365: Cell injury/death leads to Neuroinflammation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	adjacent	Moderate	
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	Moderate	
Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence

Sex	Evidence
Male Female	

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

Neuroinflammation has been described in mammals (rat, mouse, monkey, human).

Key Event Relationship Description

The pioneering work of Kreutzberg and coworkers (1995, 1996) has shown that neuronal injury leads to neuroinflammation, with microglia and astrocyte reactivities. Several chemokines and chemokines receptors (fraktalkine, CD200) control the neuron-microglia interactions, and a loss of this control can trigger microglial reactivity (Blank and Prinz, 2013; Chapman et al., 2000; Streit et al., 2001). Upon injury causing neuronal death (mainly necrotic), signals termed Damage-Associated Molecular Patterns (DAMPs) are released by damaged neurons and promote microglial reactivity (Marin-Teva et al., 2011; Katsumoto et al., 2014). Toll-like receptors (TLRs) are pattern-recognition receptors that recognize specific pathogen- and danger-associated molecular signatures (PAMPs and DAMPs) and subsequently initiate inflammatory and immune responses. Microglial cells express TLRs, mainly TLR-2, which can detect neuronal cell death (for review, see Hayward and Lee, 2014). TLR-2 functions as a master sentry receptor to detect neuronal death and tissue damage in many different neurological conditions including nerve trans-section injury, traumatic brain injury and hippocampal excitotoxicity (Hayward and Lee, 2014). Astrocytes, the other cellular mediator of neuroinflammation (Ranshoff and Brown, 2012) are also able to sense tissue injury via TLR-3 (Farina et al., 2007; Rossi, 2015).

Evidence Supporting this KER

Biological Plausibility

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

Empirical Evidence

Include consideration of temporal concordance here

Pb

In 3D cultures prepared from fetal rat brain cells exposed to Pb (10^{-6} - 10^{-4} M for 10 days), Pb-induced neuronal death was evidenced by a decrease of cholinergic and GABAergic markers associated to a decrease in protein content, and was accompanied by microglial and astrocyte reactivities (Zurich et al., 2002). These effects were more pronounced in immature than in differentiated cultures (Zurich et al., 2002). In adult rats, exposure to 100 ppm of Pb for 8 weeks caused neuronal death, evidenced by an increase in apoptosis (TUNEL) that was associated with microglial reactivity and an increase in IL-1b, TNF-a and i-NOS expression (Liu et al., 2012). Acute exposure to Pb (25 mg/kg, ip, for 3 days) increased GFAP and glutamate synthetase expression with impaiment of glutamate uptake and probable neuronal injury (Struzynska, 2000; Struzynska et al., 2001).

It is interesting to note that glial cells and in particular astrocytes are able to accumulate lead, suggesting that thes cells may be also a primary target of lead neurotoxic effects (Zurich et al., 1998; Lindhal et al., 1999).

Domoic acid

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

Mercury

Young mice receiving a fish diet (MeHgCl) for 3 months exhibited in cortex a decrease of the chemokine Ccl_2 and neuronal death, as measured by a decrease in cell density, as well as microglial reactivity (increase in Iba1-labelled cells) (**Godefroy et al., 2012**)

Perinatal exposure to MeHgCl (GD7-PD21, 0.5 mg/kg bw/day in drinking water) lead to a delayed decrease (PD 36) of cholinergic muscarinic receptors in cerebellum accompanied by astrogliosis (**Roda et al., 2008**).

Immature rat brain cell cultures maintained in 3D conditions were exposed to either MeHgCl or $HgCl_2$ (10^{-9} – 10^{-6} M, for 10 days). This treatment caused microglial and astrocyte activation without neuronal death, but a reversible decrease of the expression of the neuronal marker MAP2 (**Monnet-Tschudi et al., 1996 ; Eskes et al., 2002**).

Adult marmoset exposed acutely to 5 mg Hg/kg/day p.o. exhibited apoptosis in occipital cortex, as well as glial reactivity (GFAP and Iba1 increased). Mercury content in occipital cortex was 31 mg/g (**Yamamoto et al., 2012**).

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Human LUHMES cells as model of dopaminergic neurons and the human astrocyte cell line CFF-STTG1 were exposed to MeHgCl (0.25 -5 mM), thiomersal (0.25 – 5 mM) or HgCl2 (5-35 mM), what affected their cell viability. Neurons were much more sensitive than astrocytes (**Lohren et al., 2015**).

A direct activation of rat primary microglial cells and astrocytes was observed after exposure to MeHgCl (10^{-10}-10^{-6} M, for 5 days). (**Eskes et al., 2002**).

Astrocyte + microglia in co-cultures exposed to mercury (1-5 mM for 30 min to 6 days) showed lower levels of GSH in microglia than in astrocytes (**Ni et al., 2011 ; 2012**).

Human primary astrocyte cell line exposed to MeHgCl (1.125 mM) for 24h and 72h did not exhibit an increase of GFAP, but of NfkB after the 72h (**Malfa et al., 2014**).

Sex dependency

In prairie voles 10 weeks exposure to 600 ppm $HgCl_2$ in drinking water lead to an increase of TNF-a in hippocampus of male, but not in female (**Curtis et al., 2011**).

Acrylamide (acrylamide is a common food contaminant generated by heat processing)

Adult mice received 10, 20, 30 mg/kg bw for 4 weeks. The dose of 20 mg/kg bw caused neurological symptoms (ex. cognitive impairment) associated to an increased oxidative stress, a decrease of GSH and glial reactivity (GFAP and Iba1 increased) in cortex, hippocampus and striatum. An increase in TNF-a, IL-1b and i-NOS expression in all 3 brain regions was also observed. (**Santhanasabepathy et al., 2015**)

Isolated and/or co-cultures of microglial cells or astrocytes treated with acrylamide 0-1mM for 24-96h exhibited an increased release of TNF-a, IL-1b, IL-6 and G-CSF, suggesting a direct effect of acrylamide on glial cells (**Zhao et al., 2017a,b,c**).

Neonatal rat astrocytes treated with acrylamide (0.1-1mM) for 7, 11, 15, or 20 days increased their proliferation rate as measured by PCNA staining. Astrocyte proliferation is also a sign of reactivity. (**Aschner et al., 2005)**.

Uncertainties and Inconsistencies

Pb

It is interesting to note that glial cells and in particular astrocytes are able to accumulate lead, suggesting that thes cells may be also a primary target of lead neurotoxic effects (Zurich et al., 1998; Lindhal et al., 1999).

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

Pb decreased IL-6 secretion by isolated astrocytes (Qian et al., 2007). Such a decrease was also observed in isolated astrocytes treated with methylmercury, and was reverted in microglia astrocyte co-cultures, suggesting that cell-cell interactions can modify the response to a toxicant and that cultures of a single cell type may not be representative of the organ toxicity (Eskes et al., 2002).

Domoic acid

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

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

Mercury

Mouse developmental exposure to 50 mM of $HgCl_2$ in maternal drinking water from GD8 to PD21 did not induce any change in GM-CSF, IFN-g, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70. IL-13, IL-17, MCP1, MIP2 and TNF-a measured by Luminex in brain slices of PD21 and PD70. No sex differences, but brain increase of IgG and increased sociability in females (Zhang et al., 2012).

3D rat brain cell cultures treated for 10 days with HgCl2 or MeHgCl (10-10 - 10-6 M) exhibited increased apotosis measured by TUNEL, but exclusively in immature cultures. The proportion of cells undergoing apoptotis was highest for astrocytes than for neurons. But the apoptotic nuclei were not associated with reactive microglial cells as evidenced by double staining (Monnet-Tschudi, 1998).

Acrylamide

A 2 weeks exposure to acrylamide in drinking water (44mg/kg/day) induced behavioral effects, such a decreased in locomotor activity, but with no effect at gene level on neuronal and inflammatory markers analyzed in somatosensory and motor cortex (Bowyer et al., 2009).

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Relationship: 1718: Cell injury/death leads to Tissue resident cell activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	Moderate	
Protein Alkylation leading to Liver Fibrosis	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Liver:

Human [Winwood and Arthur,1993; Roberts et al., 2007; Kolios et al., 2006]

Rat [Tukov et al., 2006; Roberts et al., 2007]

Key Event Relationship Description

The pioneering work of Kreutzberg and coworkers (1995, 1996) has shown that neuronal injury leads to neuroinflammation, with microglia and astrocyte reactivity. Several chemokines and chemokines receptors (fraktalkine, CD200) control the neuron-microglia interactions, and a loss of this control can trigger microglial reactivity (Blank and Prinz, 2013; Chapman et al., 2000; Streit et al., 2001). Upon injury causing neuronal death (mainly necrotic), signals termed Damage-Associated Molecular Patterns (DAMPs) are released by damaged neurons and promote microglial reactivity (Marin-Teva et al., 2011; Katsumoto et al., 2014). Toll-like receptors (TLRs) are pattern-recognition receptors that recognize specific pathogen- and danger-associated molecular signatures (PAMPs and DAMPs) and subsequently initiate inflammatory and immune responses. Microglial cells express TLRs, mainly TLR-2, which can detect neuronal cell death (for review, see Hayward and Lee, 2014). TLR-2 functions as a master sentry receptor to detect neuronal death and tissue damage in many different neurological conditions including nerve trans-section injury, traumatic brain injury and hippocampal excitotoxicity (Hayward and Lee, 2014). Astrocytes, the other cellular mediator of neuroinflammation (Ranshoff and Brown, 2012) are also able to sense tissue injury via TLR-3 (Farina et al., 2007; Rossi, 2015).

LIVER:

Damaged hepatocytes release reactive oxygen species (ROS), cytokines such as TGF-β1 and TNF-α, and chemokines which lead to oxidative stress, inflammatory signalling and finally activation of the resident macrophages in the liver, Kupffer cells (KCs). ROS generation in hepatocytes results from oxidative metabolism by NADH

oxidase (NOX) and cytochrome 2E1 activation as well as through lipid peroxidation. Damaged liver cells trigger a sterile inflammatory response with activation of innate immune cells through release of damage-associated molecular patterns (DAMPs), which activate KCs through toll-like receptors and recruit activated neutrophils and monocytes into the liver. Central to this inflammatory response is the promotion of ROS formation by these phagocytes. Upon initiation of apoptosis hepatocytes undergo genomic DNA fragmentation and formation of apoptotic bodies; these apoptotic bodies are consecutively engulfed by KCs and cause their activation. This increased phagocytic activity strongly up-regulates NOX expression in KCs, a superoxide producing enzyme of phagocytes with profibrogenic activity, as well as nitric oxide synthase (iNOS) mRNA transcriptional levels with consequent harmful reaction between ROS and nitricoxide (NO), like the generation of cytotoxic peroxinitrite (N2O3). ROS and/or diffusible aldehydes also derive from liver sinusoidal endothelial cells (LSECs) which are additional initial triggers of KC activation. [Winwood and Arthur,1993; Luckey and Petersen, 2001; Roberts et al., 2007; Malhi, H. et al., 2010; Canbay et al., 2004; Orrenius et al., 2012; Kisseleva and Brenner, 2008; Jaeschke, 2011; Li et al., 2008; Poli, 2000]

Evidence Supporting this KER

LIVER:

There is convincing theoretical evidence that hepatocyte injury and apoptosis causes KC activation, as well as inflammation and oxidative stress. But there are only limited experimental studies which could show that there is a direct relationship between these two events with temporal concordance. Specific markers for activated KCs have not been identified yet. KC activation cannot be detected morphologically by staining techniques since cell morphology does not change, but cytokines release can be measured (with the caveat that KCs activate spontaneously in vitro) and used as marker for KC activation. [Canbay et al., 2003; Soldatow et al., 2013] Tukov et al. examined the effects of KCs cultured in contact with rat hepatocytes. They found that by adding KCs to the cultures they could mimic *in vivo* drug-induced inflammatory responses. Experiments on cells of the macrophage lineage showed significant aldehyde-induced stimulation of the activity of protein kinase C, an enzyme involved in several signal transduction pathways. Further, 4-Hydroxynonenal (HNE) was demonstrated to up-regulate TGF-β1 expression and synthesis in isolated rat KCs. [Tukov et al., 2006] Canbay et al could prove that engulfment of hepatocyte apoptotic bodies stimulated KC generation of cytokines. [LeCluyse et al., 2012]

Biological Plausibility

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

Empirical Evidence

Mercury

Young mice receiving a fish diet (MeHgCl) for 3 months exhibited in cortex a decrease of the chemokine Ccl_2 and neuronal death, as measured by a decrease in cell density, as well as microglial reactivity (increase in Iba1-labelled cells) (Godefroy et al., 2012)

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Immature rat brain cell cultures maintained in 3D conditions were exposed to either MeHgCl or $HgCl_2$ (10^{-9} – 10^{-6} M, for 10 days). This treatment caused microglial and astrocyte activation without neuronal death, but a reversible decrease of the expression of the neuronal marker MAP2 (Monnet-Tschudi et al., 1996 ; Eskes et al., 2002).

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A direct activation of rat primary microglial cells and astrocytes was observed after exposure to MeHgCl (10^{-10}-10^{-6} M, for 5 days). (Eskes et aé., 2002).

Astrocyte + microglia in co-cultures exposed to mercury (1-5 mM for 30 min to 6 days) showed lower levels of GSH in microglia than in astrocytes (Ni et al., 2011 ; 2012).

Human primary astrocyte cell line exposed to MeHgCl (1.125 mM) for 24h and 72h did not exhibit an increase of GFAP, but of NfkB after the 72h (Malfa et al., 2014).

Human mast cells (leukemic LAD2, derived from umbilical cord blood) showed an increase of IL-6 release when exposed to $HgCl_2$ (0.1-10 mM, for 10 min to 24h). It is hypothesized that mast cell activation could lead to BBB disruption and to neuroinflammation. (Kempurai et al., 2010).

Sex dependency

In prairie voles 10 weeks exposure to 600 ppm $HgCl_2$ in drinking water lead to an increase of TNF-a in hippocampus of male, but not in female (Curtis et al., 2011).

Acrylamide (acrylamide is a common food contaminant generated by heat processing)

Adult mice received 10, 20, 30 mg/kg bw for 4 weeks. The dose of 20 mg/kg bw caused neurological symptoms (ex. cognitive impairment) associated to an increased oxidative stress, a decrease of GSH and glial reactivity (GFAP and Iba1 increased) in cortex, hippocampus and striatum. An increase in TNF-a, IL-1b and i-NOS expression in all 3 brain regions was also observed. (Santhanasabepathy et al., 2015)

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Neonatal rat astrocytes treated with acrylamide (0.1-1mM) for 7, 11, 15, or 20 days increased their proliferation rate as measured by PCNA staining. Astrocyte proliferation is also a sign of reactivity. (Aschner et al., 2005).

Acrolein

Adult rat received an infusion of acrolein (15, 50, 150 nmoles/0.5 ml) directly in substantia nigra which caused a decrease of Tyrosine hydroxylase immunostaining, an increase in caspase 1 and an activation of microglial cells and astrocytes (Wang et al., 2017).

Similar treatment as above induced an increase in lipid peroxidation, of hsp32 and of caspase 1 with an increase in GFAP and in ED1 (marker of macrophagic microglial cells) as well as of IL-1b (Zhao et al., 2017).

Uncertainties and Inconsistencies

Mercury

Mouse developmental exposure to 50 mM of $HgCl_2$ in maternal drinking water from GD8 to PD21 did not induce any change in GM-CSF, IFN-g, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70. IL-13, IL-17, MCP1, MIP2 and TNF-a measured by Luminex in brain slices of PD21 and PD70. No sex differences, but brain increase of IgG and increased sociability in females (Zhang et al., 2012).

3D rat brain cell cultures treated for 10 days with HgCl2 or MeHgCl (10-10 - 10-6 M) exhibited increased apotosis measured by TUNEL, but exclusively in immature cultures. The proportion of cells undergoing apoptotis was highest for astrocytes than for neurons. But the apoptotic nuclei were not associated with reactive microglial cells as evidenced by double staining (Monnet-Tschudi, 1998).

Acrylamide

A 2 weeks exposure to acrylamide in drinking water (44mg/kg/day) induced behavioral effects, such a decreased in locomotor activity, but with no effect at gene level on neuronal and inflammatory markers analyzed in somatosensory and motor cortex (Bowyer et al., 2009).

LIVER:

The detailed mechanisms of the KC - hepatocyte interaction and its consequences for both normal and toxicant-driven liver responses remain to be determined. KC activation followed by cytokine release is associated in some cases with evident liver damage, whereas in others this event is unrelated to liver damage or may be even protective; apparently this impact is dependent on the quantity of KC activation; excessive or prolonged release of KC mediators can switch an initially protective mechanism to a damaging inflammatory response. Evidence suggests that low levels of cytokine release from KCs constitute a survival signal that protects hepatocytes from cell death and in some cases, stimulates proliferation. [Roberts et al., 2007]

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LIVER:

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Relationship: 1687: Neuroinflammation leads to Cell injury/death

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	Moderate	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Male	
Female	

Most experimental evidences derived from mouse and rat studies.

Key Event Relationship Description

Cells of the innate (microglia and astrocytes) and of the adaptive (infiltrating monocytes and lymphocytes) immune system of the brain have various ways to kill neighboring cells. This is in part due to evolutionary-conserved mechanisms evolved to kill virus-infected cells or tumor cells; in part it is a bystander phenomenon due to the release of mediators that should activate other cells and contribute to the killing of invading micro-organisms. An exaggerated or unbalanced activation of immune cells can thus lead to parenchymal (neuronal) cell death (Gehrmann et al., 1995). Mediators known to have such effects comprise components of the complement system and cytokines/death receptor ligands triggering programmed cell death (Dong and Benveniste, 2001). Various secreted proteases (e.g. matrix metalloproteases), lipid mediators (e.g. ceramide or gangliosides) or reactive oxygen species can contribute to bystander death of neurons (Chao et al., 1995; Nakajima et al., 2002; Brown and Bal-Price, 2003; Kraft and Harry, 2011; Taetzsch and Block, 2013). The equimolar production of superoxide and NO from glial cells can lead to high steady levels of peroxynitrite, which is a very potent cytotoxicant (Yuste et al., 2015). Already stressed neurons, with an impaired anti-oxidant defence system, are more sensitive to such mediators (Xu et al., 2015). Healthy cells continuously display anti "eat-me" signals, while damaged and stressed neurons/neurites display "eat-me" signals that may be recognized by microglia as signals to start phagocytosis (Neher et al., 2012) or by astrocytes (Wakida et al., 2018; Byun and Chung, 2018; Gomez-Arboledas et al., 2018; Morizawa et al., 2017). Reactive astrocytes are also able to release neurotoxic molecules (Mena and Garcia de Ybenes, 2008; Niranjan, 2014). However, astrocytes may also be protective due to their capacity to quench free radicals and secrete neurotrophic factors. The activation of astrocytes may reduce neurotrophic support to neurons (for review, Mena and Garcia de Ybenes, 2008).

Evidence Supporting this KER

Biological Plausibility

In vitro co-culture experiments have demonstrated that reactive glial cells (microglia and astrocytes) can kill neurons (Chao et al., 1995; Brown and Bal-Price, 2003; Kraft and Harry, 2011; Taetzsch and Block, 2013) and that interventions with e.g. i-NOS inhibition can rescue the neurons (Yadav et al., 2012; Brzozowski et al., 2015). Drugs that block Toll like receptor pathways, which are expressed by glial cells have been proven to be protective by decreasing ROS and RNS production (Lucas et al., 2013).

Reactive microglia can remove synapses, a process known as synapse stripping (Banati et al., 1993; Kettenmann et al., 2013). Reactive astrocytes were also associated with neurite and synapse reduction (Calvo-Ochoa et al., 2014). Microglia can modulate synapse plasticity, an effect mediated by cytokines. During development, microglia can promote synaptogenesis or engulf synapses, a process known as synaptic pruning (for review, Jebelli et al., 2015). It is hypothesized that alterations in microglia functioning during synapse formation and maturation of the brain can have significant long-term effects on the final established neural circuits (for review, Harry and Kraft, 2012). The fact that astrocytes can receive and respond to the synaptic information produced by neuronal activity, owing to their expression of a wide range of neurotransmitter receptors, has given rise to the concept of tripartite synapse (for review, Perez-Alvarez and Araque, 2013; Bezzi and Volterra, 2001). Pro-inflammatory cytokines, such as TNF-a, IL-1b and IL-6, which are produced by reactive astrocytes, are on one side implicated in synapse formation and scaling, long-term potentiation and neurogenesis (for review, Bilbo and Schwartz, 2009) and on the other side can kill neurons (Chao et al., 1995; Kraft and Harry, 2011). Taken together, this suggests that neuron-glia interactions are tightly regulated and that an imbalance, such as increased or long-term release of these inflammatory mediators may lead to deleterious effects on neurons.

Empirical Evidence

Mercury

Mercury accumulates in the brain particularly in astrocytes and induce astrocyte swelling, excitatory amino acid release and decreased anti-oxidant protections (Shanker et al., 2003; Allen et al., 2001), features that are also observed in reactive astrocytes. Due to the central role of astrocytes for neuronal function (control of water transport, production of trophic factors, of anti-oxidants, tri-partite synapse,… (Ximeres da Silva, 2016; Bezzi and Volterra, 2001; Hertz and Zielke, 2004; Sidoryk-Wegrzynowicz et al., 2011), it is thought that neuronal dysfunction may be secondary to disturbance in astrocytes (Aschner et al., 2007).

Perinatal exposure (GD7-PD21) of rat to MeHgCl (0.5 mg/kg bw/day) in drinking water lead to gliosis in cerebellum of immature rats (PD21) without affecting the cholinergic system. In contrast, at PD36, astrogliosis was accompanied by an increase of muscarinic M2-immunopositive Bergman cells and a lack of M3 muscarinic receptors in the molecular layer. These results suggest that astrogliosis which is observed first at PD21 may be responsible of the delayed effects of mercury on neurons (**Roda et al., 2008**).

Developmental exposure of mice from GD8 to PD21 to 50 mM $HgCl_2$ in maternal drinking water: Female offsprings exhibited higher neuroinflammation which is associated with altered social behavior (**Zhang et al., 2013**).

MG17, a novel triazole derivative, was able to reduce mercury-induced upregulation of IL-1b, IL-6 and TNF-a (measured by RT-PCR) and proved to be protective against mercury-induced neurodegeneration (**Matharasala et al., 2017**).

Adult rats exposed to MeHg (5mg/kg bw) for 12 consecutive days exhibited piknotic nuclei in cerebellar granule cells, what was reverted by a co-administration of CA074 an inhibitor of cathepsin released by activated microglia. These observations strongly suggest that the mercury–induced neuronal pathological changes are secondary to microglial activation (**Sakamoto et al., 2008**).

Acrylamide

Rats exposed to acrylamide (20 mg/kg bw for 4 weeks) together with farmesol (sequiterpene) showed a downregulation of astrogliosis (i.e. decreased GFAP) and of microgliosis (i.e. decreased Iba1) and of TNF-a, Il-1b and i-NOS in cortex, hippocampus and striatum. This was associated with a marked improvement in motor coordination and a decrease in markers of oxidative stress, as compared to rats exposed to acrylamide alone (**Santhanasabapathy et al., 2015**).

Uncertainties and Inconsistencies

In 3D rat brain cell-cultures, co-administration of the pro-inflammatory cytokine IL-6 (10 ng/ml) together with non-cytotoxic concentrations of MeHgCl (3×10^{-7} M) for 10 days protected from the mercury-induced decreased in MAP2 immunostaining, suggesting a positive effect of IL-6, in accord with its descibed trophic activity (**Eskes et al., 2002**).

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Relationship: 1719: Increased pro-inflammatory mediators leads to Cell injury/death

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	Moderate	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development, adulthood and aging	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Cells of the innate (microglia and astrocytes) and of the adaptive (infiltrating monocytes and lymphocytes) immune system of the brain have various ways to kill neighboring cells. This is in part due to evolutionary-conserved mechanisms evolved to kill virus-infected cells or tumor cells; in part it is a bystander phenomenon due to the release of mediators that should activate other cells and contribute to the killing of invading micro-organisms. An exaggerated or unbalanced activation of immune cells can thus lead to parenchymal (neuronal) cell death (Gehrmann et al., 1995). Mediators known to have such effects comprise components of the complement system and cytolkines/death receptor ligands triggering programmed cell death (Dong and Benveniste, 2001). Various secreted proteases (e.g. matrix metalloproteases), lipid mediators (e.g. ceramide or gangliosides) or reactive oxygen species can contribute to bystander death of neurons (Chao et al., 1995; Nakajima et al., 2002; Brown and Bal-Price, 2003; Kraft and Harry, 2011; Taetsch and Block, 2013). The equimolar production of superoxide and NO from glial cells can lead to high steady levels of peoxynitrite, which is a very potent cytotoxicant (Yuste et al., 2015). Already stressed neurons, with an impaired anti-oxidant defence system, are more sensitive to such mediators (Xu et al., 2015). Healthy cells continuously display anti "eat-me" signals, while damaged and stressed neurons/neurites display "eat-me" signals that may be recognized by microglia as signals to start phagocytosis (Neher et al., 2012). Reactive astrocytes are also able to release neurotoxic molecules (Mena and Garcia de Ybenes, 2008; Niranjan, 2014). However, astrocytes may also be protective due to their capacity to quench free radicals and secrete neurotrophic factors. The activation of astrocytes may reduce neurotrophic support to neurons (for review, Mena and Garcia de Ybenes, 2008).

Evidence Supporting this KER

Biological Plausibility

In vitro co-culture experiments have demonstrated that reactive glial cells (microglia and astrocytes) can kill neurons (Chao et al., 1995; Brown and Bal-Price, 2003; Kraft and Harry, 2011; Taetzsch and Block, 2013) and that interventions with e.g. i-NOS inhibition can rescue the neurons (Yadav et al., 2012; Brzozowski et al., 2015). Drugs that block Toll like receptor pathways, which are expressed by glial cells have been proven to be protective by decreasing ROS and RNS production (Lucas et al., 2013).

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Empirical Evidence

Mercury

Mercury accumulates in the brain particularly in astrocytes and induce astrocyte swelling, excitatory amino acid release and decreased anti-oxidant protections (Shanker et al., 2003; Allen et al., 2001), features that are also observed in reactive astrocytes. Due to the central role of astrocytes for neuronal function (control of water transport, production of trophic factors, of anti-oxidants, tri-partite synapse,… (Ximeres da Silva, 2016; Bezzi and Volterra, 2001; Hertz and Zielke, 2004; Sidoryk-Wegrzynowicz et al., 2011), it is thought that neuronal dysfunction may be secondary to disturbance in astrocytes (Aschner et al., 2007).

Perinatal exposure (GD7-PD21) of rat to MeHgCl (0.5 mg/kg bw/day) in drinking water lead to gliosis in cerebellum of immature rats (PD21) without affecting the cholinergic system. In contrast, at PD36, astrogliosis was accompanied by an increase of muscarinic M2-immunopositive Bergman cells and a lack of M3 muscarinic receptors in the molecular layer. These results suggest that astrogliosis which is observed first at PD21 may be responsible of the delayed effects of mercury on neurons (Roda et al., 2008).

Developmental exposure of mice from GD8 to PD21 to 50 mM $HgCl_2$ in maternal drinking water: Female offsprings exhibited higher neuroinflammation which is associated with altered social behavior (Zhang et al., 2013).

MG17, a novel triazole derivative, was able to reduce mercury-induced upregulation of IL-1b, IL-6 and TNF-a (measured by RT-PCR) and proved to be protective against mercury-induced neurodegeneration (Matharasala et al., 2017).

Adult rats exposed to MeHg (5mg/kg bw) for 12 consecutive days exhibited piknotic nuclei in cerebellar granule cells, what was reverted by a co-administration of CA074 an inhibitor of cathepsin released by activated microglia. These observations strongly suggest that the mercury–induced neuronal pathological changes are secondary to microglial activation (Sakamoto et al., 2008).

Acrylamide

Rats exposed to acrylamide (20 mg/kg bw for 4 weeks) together with farmesol (sequiterpene) showed a downregulation of astrogliosis (i.e. decreased GFAP) and of microgliosis (i.e. decreased Iba1) and of TNF-a, Il-1b and i-NOS in cortex, hippocampus and striatum. This was associated with a marked improvement in motor coordination and a decrease in markers of oxidative stress, as compared to rats exposed to acrylamide alone (Santhanasabapathy et al., 2015).

Uncertainties and Inconsistencies

In 3D rat brain cell-cultures, co-administration of the pro-inflammatory cytokine IL-6 (10 ng/ml) together with non-cytotoxic concentrations of MeHgCl (3×10^{-7} M) for 10 days protected from the mercury-induced decreased in MAP2 immunostaining, suggesting a positive effect of IL-6, in accord with its descibed trophic activity (Eskes et al.,

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Relationship: 1688: Cell injury/death leads to Neuronal network function, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	Moderate	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Male	
Female	

Support for the link between cell injury/death and decreased neuronal network formation and function can be found in rat, mouse and minnow. (for references, see empirical evidences)

Key Event Relationship Description

Under physiological conditions, in the developing nervous system, apoptosis occurs during the process of synaptogenesis, where competition leads to the loss of excess neurons and to the connection of the appropriate neurons (Buss et al., 2006; Mennerick and Zorumski, 2000; Oppenheim, 1991). When a stressor increases the number of apoptotic cells this KE has a negative effect on synaptogenesis as the reduced number of neurons (besides the ones that have been already eliminated through the physiological process of apoptosis) provides limited dendritic fields for receiving synaptic inputs from incoming axons. At the same time the loss of neurons also means that there are less axons to establish synaptic contacts (Olney, 2014), leading to reduced synaptogenesis. The ability of a neuron to communicate is based on neural network formation that relies on functional synapse establishment (Colón-Ramos, 2009). The main roles of synapses are the regulation of intercellular communication in the nervous system, and the information flow within neural networks. The connectivity and functionality of neural networks depends on where and when synapses are formed. Therefore, the decreased synapse formation due to cell death during the process of synaptogenesis is critical and leads to decrease of neural network formation and function in the adult brain.

Synaptic transmission and plasticity require the integrity of the anatomical substrate. The connectivity of axons emanating from one set of cells to post-synaptic side of synapse on the dendrites of the receiving cells must be intact for effective communication between neurons. Changes in the placement of cells within the network due to delays in neuronal migration, the absence of a full formation of dendritic arbors and spine upon which synaptic contacts are made, and the lagging of transmission of electrical impulses due to insufficient myelination will individually and cumulatively impair synaptic function.

Therefore, chemicals inducing neuronal cell death by apoptosis or necrosis, or interfering with a particular system of neurotransmitters, will alter network structure and function.

Evidence Supporting this KER

Biological Plausibility

Recently, Dekkers et al. 2013 have reviewed how under physiological conditions components of the apoptotic machinery in developing brain regulate synapse formation and neuronal connectivity. For example, caspase activation is known to be required for axon pruning during development to generate neuronal network (reviewed in Dekkers et al., 2013). Experimental work carried out in Drosophila melanogaster and in mammalian neurons shows that components of apoptotic machinery are involved in axonal degeneration that can consequently interfere with synapse formation (reviewed in Dekkers et al., 2013). Furthermore, Bax mutant mice studies indicate that the lack of this pro-apoptotic protein BAX leads to disruption of intrinsically photosensitive retinal ganglion cells spacing and dendritic stratification that affects synapse localization and function (Chen et al., 2013).

Neuronal network formation and function are established via the process of synaptogenesis. The developmental period of synaptogenesis is critical for the formation of the basic circuitry of the nervous system, although neurons are able to form new synapses throughout life (Rodier, 1995). The brain electrical activity dependence on synapse formation is critical for proper neuronal communication.

Alterations in synaptic connectivity lead to refinement of neuronal networks during development (Cline and Haas, 2008). Indeed, knockdown of PSD-95 arrests the functional and morphological development of glutamatergic synapses (Ehrlich et al., 2007).

Studies of the last 30 years demonstrated that astrocytes possess functional receptors for neurotransmitters and respond to their stimulation via release of gliotransmitters, including glutamate. These findings have led to a new concept of neuron–glia intercommunication where astrocytes play an unsuspected dynamic role by integrating neuronal inputs and modulating synaptic activity (Rossi and Volterra, 2009). According to the concept termed "tripartite synapse", the emerging view is that brain function actually arises from the coordinated activity of a network comprising both neurons and astrocytes. Furthermore, myelinating glial cells are well-known to insulate axons and to speed up action potential propagation. Be it motor skill learning or social behaviors in higher vertebrates, proper myelination is critical in shaping brain functions. Neurons rely on their myelinating partners not only for setting conduction speed, but also for regulating the ionic environment and fueling their energy demands with metabolites. Also, long-term axonal integrity and neuronal survival are maintained by oligodendrocytes and loss of this well-coordinated axon-glial interplay contributes to brain diseases (Saab and Nave, 2017). Therefore, reduction in glial cell number and/or reduction in myelination of axons, will very much impact the neural network function.

Empirical Evidence

Mercury

KE_{up} Cell injury/death	KE_{down} Decreased network formation and function	species; developmental stage of exposure to stressor	Stressor	Dose or conc. Duration	Protective/ aggravating evidence	Reference
Apoptosis measured by levels of Cleaved caspase3 (2x CTR values)	Inhibition of hippocampal-dependent memory processes at P35	Rat	MeHgCl	5 µg g^{-1} single injection		Falluel-Morel, 2007

	(water maze)	exposed at P7				
Apoptosis measured by DNA laddering and electron microscopy	Nerve fibers degeneration in peripheral nerves, sensory ganglia, root nerve, spinal cord and cerebellum	Rat adult exposure	MeHgCl	4-10 mg kg^{-1} day^{-1} 7-20 days subcutaneous or oral		Nagashima, 1997 (review)
Apoptosis measured by in situ DNA strand breaks, DNA laddering and electron microscopy	Nerve fibers degeneration in cerebellum	Rat adult exposure	MeHgCl	4 mg kg^{-1} day^{-1} 20 days oral		Nagashima, 1996
Necrosis and apoptosis measured by chromatin condensation on primary cultures of cortical neurons prepared from the F1 generation pups	Fragmentation of the neuronal network (microtubule disruption) in vitro and long-term memory impairment in vivo (at P90)	Rat pregnant exposed to mercury at GD15	MeHgCl	4 and 8 mg kg^{-1} single gavage		Ferraro, 2009
Extensive neuronal cell loss (histopathology) in F1 generation pups (PND25)	Decreased activity of acetylcholinesterase in F1 generation pups (PND24) and less time latency to fall in rotarod test, increased escape time latency in Morris water maze test, increased immobility time in forced-swim test	Rat pregnant exposed to mercury from GD5 till parturition	MeHgCl	1.5 mg kg^{-1} orally	Co-administration of fisetin (plant flavonoid) alleviated all MeHgCl effects	Jacob, 2017
Apoptosis observed 7 days after exposure	Degeneration of the dopaminergic system observed 7 days after exposure	Rat adult exposure	Acrolein	Single intranigral infusion of 15, 50, 150 nmoles		Wang, 2017

Acrylamide

No publications found to support this KE

Uncertainties and Inconsistencies

Ogawa et al. (2011) reported decreased apoptosis and an increase in the number of Gabaergic interneurons in the dentate gyrus of Sprague-Dawley pups either maternally exposed to acrylamide or directly injected with acrylamide.

Although it appears evident that a decrease in cell number, in dendritic arborization or in axonal growth, as well as synapse alterations may lead to decreased neuronal network formation and function, the exact mechanism remain to be elucidated.

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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	

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 2nd 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: 1690: Oxidative Stress leads to Cell injury/death

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
zebra fish	Danio rerio	High	NCBI
salmonid fish	salmonid fish	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	
Female	

Rat, Mouse: (Sarafian *et al.*, 1994; Castoldi *et al.*, 2000; Kaur *et al.*, 2006; Franco *et al.*, 2007; Lu *et al.*, 2011; Polunas *et al.*, 2011)

(Richetti *et al.*, 2011) - Adult and healthy zebrafish of both sexes (12 animals and housed in 3 L) mercury chloride final concentration of 20 mg/L. Mercury chloride promoted a significant decrease in acetylcholinesterase activity and the antioxidant competence was also decreased.

(Berntssen, Aatland and Handy, 2003) - Atlantic salmon (*Salmo salar L.*) were supplemented with mercuric chloride (0, 10, or 100 mg Hg per kg) or methylmercury chloride (0, 5, or 10 mg Hg per kg) for 4 months.

Methylmercury chloride

- accumulated significantly in the brain of fish fed 5 or 10 mg/kg
- No mortality or growth reduction
- - 2-fold increase in the antioxidant enzyme super oxide dismutase (SOD) in the brain
- 10 mg/kg - 7-fold increase of lipid peroxidative products (thiobarbituric acid reactive substances, TBARS) and a subsequently 1.5-fold decrease in anti oxidant enzyme

activity (SOD and glutathione peroxidase, GSH-Px). Fish also had pathological damage (vacoulation and necrosis), significantly reduced neural enzyme activity (5-fold reduced monoamine oxidase, MAO, activity), and reduced overall post-feeding activity behaviour.

Mercuric chloride

- accumulated significantly in the brain only at 100 mg/kg
- No mortality or growth reduction
- 100 mg/kg - significant reduced neural MAO activity and pathological changes (astrocyte proliferation) in the brain, however, neural SOD and GSH-Px enzyme activity, lipid peroxidative products (TBARS), and post feeding behaviour did not differ from controls.

Key Event Relationship Description

Oxidative stress (OS) as a concept in redox biology and medicine has been formulated in 1985 (Sies, 2015). OS is intimately linked to cellular energy balance and comes from the imbalance between the generation and detoxification of reactive oxygen and nitrogen species (ROS/RNS) or from a decay of the antioxidant protective ability. OS is characterized by the reduced capacity of endogenous systems to fight against the oxidative attack directed towards target biomolecules (Wang and Michaelis, 2010; Pisoschi and Pop, 2015). Glutathione, the most important redox buffer in cells (antioxidant), cycles between reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG), and serves as a vital sink for control of ROS levels in cells (Reynolds *et al*., 2007). Several case-control studies have reported the link between lower concentrations of GSH, higher levels of GSSG and the development of diseases (Rossignol and Frye, 2014). OS can cause cellular damage and subsequent cell death because the ROS oxidize vital cellular components such as lipids, proteins, and nucleic acids (Gilgun-Sherki, Melamed and Offen, 2001; Wang and Michaelis, 2010).

The central nervous system is especially vulnerable to free radical damage since it has a high oxygen consumption rate, an abundant lipid content and reduced levels of antioxidant enzymes (Coyle and Puttfarcken, 1993; Markesbery, 1997). It has been show that the developing brain is particularly vulnerable to neurotoxicants and OS due to differentiation processes, changes in morphology, lack of physiological barriers and less intrinsic capacity to cope with cellular stress (Grandjean and Landrigan, 2014; Sandström *et al*., 2017). However, it has to be noted that neural stem cells distinguish themselves from post-mitotic neural cells by their lower ROS levels and higher expression of the key antioxidant enzymes glutathione peroxidase. This increased "vigilance" of antioxidant mechanisms might represent an innate characteristic of NSCs, which not only defines their cell fate, but also helps them to encounter oxidative stress (Madhavan et al., 2006).

OS has been linked to brain aging, neurodegenerative diseases, and other related adverse conditions. There is evidence that free radicals play a role in cerebral ischemia-reperfusion, head injury, Parkinson's disease, amyotrophic lateral sclerosis, Down's syndrome, and Alzheimer's disease due to cellular damage (Markesbery, 1997; Gilgun-Sherki, Melamed and Offen, 2001; Wang and Michaelis, 2010). OS has also been linked to neurodevelopmental diseases and deficits like autism spectrum disorder and postnatal motor coordination deficits (Wells *et al*., 2009; Rossignol and Frye, 2014; Bhandari and Kuhad, 2015).

Evidence Supporting this KER

Biological Plausibility

A noteworthy insight, early on, was the perception that oxidation-reduction (redox) reactions in living cells are utilized in fundamental processes of redox regulation, collectively termed 'redox signaling' and 'redox control' (Sies, 2015).

Free radical-induced damage in OS has been confirmed as a contributor to the pathogenesis and patho-physiology of many chronic diseases, such as Alzheimer, atherosclerosis, Parkinson, but also in traumatic brain injury, sepsis, stroke, myocardial infraction, inflammatory diseases, cataracts and cancer (Bar-Or *et al*., 2015; Pisoschi and Pop, 2015). It has been assessed that oxidative stress is correlated with over 100 diseases, either as source or outcome (Pisoschi and Pop, 2015).

Therefore, the fact that ROS over-production can kill neurons is well accepted (Brown and Bal-Price, 2003; Taetzsch and Block, 2013). This ROS over-production can occur in the neurons themselves or can also have a glial origin (Yuste et al., 2015).

Empirical Evidence

Mercury

Oxidative stress has been implicated in the pathogenesis of methylmercury (MeHg) neurotoxicity. Studies of mature neurons suggest that the mitochondrion may be a major source of MeHg-induced reactive oxygen species and a critical mediator of MeHg-induced neuronal death, likely by activation of apoptotic pathways. (Polunas *et al*., 2011)

(Lu *et al*., 2011) - MeHg in the mouse cerebrum (in vivo) and in cultured Neuro-2a cells (in vitro).

- *In vivo* - 50µg/kg/day MeHg for 7 consecutive weeks - increased levels of lipid peroxidation in the plasma and cerebral cortex. Decreased GSH level and increase the expressions of caspase-3, -7, and -9, accompanied by Bcl-2 down-regulation and up-regulation of Bax, Bak, and p53.
- *In vitro* – 3 and 5 µM MeHg - reduced cell viability, increased oxidative stress damage, and induced several features of mitochondria-dependent apoptotic signals, including increased sub-G1 hypodiploids, mitochondrial dysfunctions, and the activation of PARP, and caspase cascades.
- These MeHg-induced apoptotic-related signals could be remarkably reversed by antioxidant NAC.

(Sarafian *et al*., 1994) - Hypothalamic mouse neural cell line GT1-7 without and with expression construct for the anti-apoptotic proto-oncogene, bcl-2.

- 3h exposure, 10 µM MeHg - increased formation of reactive ROS, and decreased levels of GSH, associated with 20% cell death. Cells transfected with an expression construct bcl-2, displayed attenuated ROS induction and negligible cell death.
- 24h exposure, 5 µM MeHg - killed 56% of control cells, but only 19% of bcl-2-transfected cells.
- By using diethyl maleate to deplete cells of GSH, we demonstrate that the differential sensitivity to MeHg was not due solely to intrinsically different GSH levels. The data suggest that MeHg-mediated cell killing correlates more closely with ROS generation than with GSH levels and that bcl-2 protects MeHg-treated cells by suppressing ROS generation.

(Castoldi *et al*., 2000) - In vitro exposure of primary cultures of rat CGCs to MeHg resulted in a time- and concentration-dependent cell death.

- 1 hr exposure, 5–10 µM MeHg - impairment of mitochondrial activity, de-energization of mitochondria and plasma membrane lysis, resulting in necrotic cell death.
- 1hr exposure, 0.5–1 µM MeHg - did not compromise cell viability, mitochondrial membrane potential and function at early time points.
- 1hr exposure, 1 µM MeHg - only a small population of neurons (+-20%) dies by necrosis. The surviving neurons show network damage, but maintain membrane integrity, mitochondrial membrane potential and function at early time points. Later, however, the cells progressively display the morphological signs of apoptosis.
- 18hr exposure, 0.5–1 µM MeHg – cells progressively underwent apoptosis reaching the 100% cell death
- insulin-like growth factor-I partially *rescued* CGCs from MeHg-triggered apoptosis.

(**Kaur,et al., 2006**) - primary cell cultures of cerebellar neurons and astrocytes from 7-day-old NMRI mice. 5 mM MeHg for 30 min.

- Twenty-one days post-astrocyte isolation - 250mM N-acetyl cysteine (NAC) or 3mM di-ethyl maleate (DEM) added to the wells 12 h prior to MeHg exposure
- 7 days post-neurons isolation - 200mM of NAC or 1.8mM of DEM added to the wells 12 h prior to MeHg exposure
- The intracellular GSH content was modified by pretreatment with NAC or DEM for 12 h.
- Treatment with 5 mM Me Hg for 30 min led to significant ($p < 0.05$) increase in ROS and reduction ($p < 0.001$) in GSH content.

- Depletion of intracellular GSH by DEM further increased the generation of MeHg-induced ROS in both cell cultures.
- NAC supplementation increased intracellular GSH and provided protection against MeHg-induced oxidative stress in both cell cultures.

(Franco *et al.*, 2007) – Mitochondrial enriched fractions from adult (2 months old) Swiss Albino male mice.

- MeHg and HgCl2 (10–100 µM) significantly decreased mitochondrial viability; this phenomenon was positively correlated to mercurial-induced glutathione oxidation.
- Both mercurials induced a significant reduction of GSH in a dose-dependent manner.
- Correlation analyses showed significant positive correlations between mitochondrial viability and glutathione content for MeHg (Pearson coefficient) 0.933; $P < 0.01$) and or HgCl2 (Pearson coefficient) 0.854; $P < 0.01$).
- Quercetin (100–300 µM) prevented mercurial-induced disruption of mitochondrial viability. Moreover, quercetin, which did not display any chelating effect on MeHg or HgCl2, prevented mercurial-induced glutathione oxidation.

(Polunas *et al.*, 2011) - Murine embryonal carcinoma (EC) cells, which differentiate into neurons following exposure to retinoic acid.

- 4h exposure, 1.5 mM MeHg - earlier and significantly higher levels of ROS production and more extensive mitochondrial depolarization in neurons than in undifferentiated EC cells. cyclosporin A (CsA) completely inhibited mitochondrial depolarization by MeHg in EC cells but only delayed this response in the neurons. In contrast, CsA significantly inhibited MeHg-induced neuronal ROS production. Cyt c release was also more extensive in neurons, with less protection afforded by CsA.

(**Sandström *et al.*, 2016**) - in vitro 3D human neural tissues from neural progenitor cells derived from human embryonic stem cells. Single MeHg exposure at day 42 of 3D culturing (week 6) and material was collected 72 h after.

- 1-10 µM - LDH activity increased, confirming induced cell death.
- 5 and 10 µM - increased HMOX1 gene expression as indirect marker of oxidative stress.

Acrylamide

(Allam *et al.*, 2011) - sixty albino *Rattus norvegicus*, 45 virgin females and 15 mature males. This study examined its effects on the development of external features in cubs.

- prenatal intoxicated group - newborns from mothers treated with ACR (10 mg/kg/day by gastric intubation) from day 7 (GD 7) of gestation till birth
- perinatal intoxicated group - newborns from mothers treated with ACR (10 mg/kg/day by gastric intubation) from GD7 of gestation till D28 after birth

ACR administered either prenatally or perinatally has been shown to induce significant retardation in the new- borns' body weights development, increase of thiobarbituric acid- reactive substances (TBARS) and oxidative stress (significant reductions in reduced glutathione, total thiols, superoxide dismutase and peroxidase activities) in the developing cerebellum. ACR treatment delayed the proliferation in the granular layer and delayed both cell migration and differentiation. Purkinje cell loss was also seen in acrylamide-treated animals. Ultrastructural studies of Purkinje cells in the perinatal group showed microvacuolations and cell loss.

(**Lakshmi *et al.*, 2012**) - Wistar male albino rats, four groups (n = 6 per group)

- II – (Acrylamide) ACR - 30 mg/kg ACR for 30 days: increase in the lipid peroxidative (LPO), protein carbonyl, hydroxyl radical and hydroperoxide levels with subsequent decrease in the activities of enzymic antioxidants and level of GSH. Cortex showed condensed nuclei along with damaged cells. Decrease in the expression of Bcl2 along with simultaneous increase in the expressions of Bax and Bad as compared to control.
- II rats – ACR + Fish oil -0.5 ml/kg b.w.fish oil orally 10 min before ACR induction with 30 mg/kg for 30 days – reversed significantly all the OS markers.

Uncertainties and Inconsistencies

Mercury-induced upregulation of GSH level and GR activity as an adaptive mechanism following lactational exposure to methylmercury (10 mg/L in drinking water) associated with motor deficit, suggesting neuronal impairment (Franco *et al.*, 2006).

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