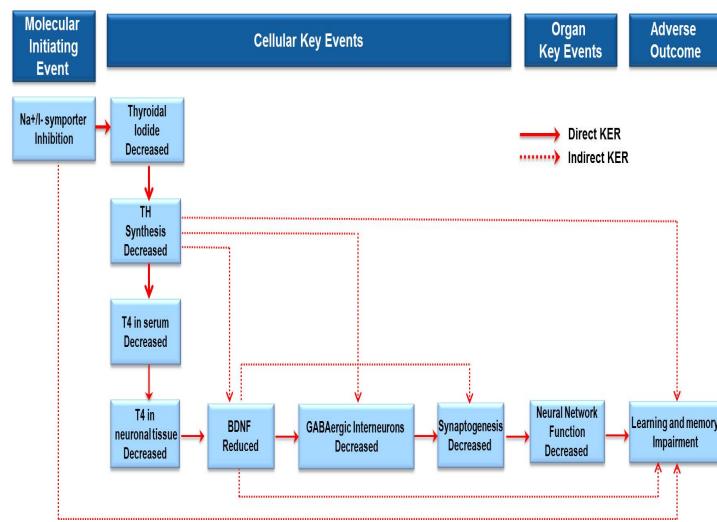


AOP 54: Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment

Short Title: NIS inhibition and learning and memory impairment

Graphical Representation**Authors**

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Abstract

The thyroid hormones (TH) are essential for brain development, maturation, and function as they regulate the early key developmental processes such as neurogenesis, cell migration, proliferation, myelination and neuronal and glial differentiation. Normal human brain development and cognitive function relies on sufficient production of TH during the perinatal period. The function of Na⁺/I⁻ symporter (NIS) is critical for the physiological production of TH levels in the serum, as it is a membrane bound glycoprotein that mediates the transport of iodide from the bloodstream into the thyroid cells, and this constitutes the initial step for TH synthesis. NIS is a well-studied target of chemicals, and its inhibition results in decreased TH synthesis and its secretion into blood leading to subsequent TH insufficiency in the brain with detrimental effects in neurocognitive function in children. The present AOP describes causative links between inhibition of NIS function (the molecular initiating event) leading to the decreased levels of TH in the blood and consequently in the brain, causing learning and memory deficit in children (Adverse outcome). Learning and memory depend upon the coordinated action of different brain regions and neurotransmitter systems creating functionally integrated neural networks. Hippocampus and cortex are the most critical brain structures involved in the process of cognitive functions in rodents and primates, including man. The overall weight of evidence for this AOP is strong. The function of NIS and its essentiality for TH synthesis is well known across species, however, quantitative information of KERs is limited.

Summary of the AOP**Events****Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)**

Sequence	Type	Event ID	Title	Short name
1	MIE	424	Inhibition, Na ⁺ /I ⁻ symporter (NIS) (https://aopwiki.org/events/424)	Inhibition, Na ⁺ /I ⁻ symporter (NIS)
2	KE	425	Decrease of Thyroidal iodide (https://aopwiki.org/events/425)	Thyroidal Iodide, Decreased
3	KE	277	Thyroid hormone synthesis, Decreased (https://aopwiki.org/events/277)	TH synthesis, Decreased
4	KE	281	Thyroxine (T4) in serum, Decreased (https://aopwiki.org/events/281)	T4 in serum, Decreased
5	KE	280	Thyroxine (T4) in neuronal tissue, Decreased (https://aopwiki.org/events/280)	T4 in neuronal tissue, Decreased
6	KE	381	Reduced levels of BDNF (https://aopwiki.org/events/381)	BDNF, Reduced
7	KE	851	Decrease of GABAergic interneurons (https://aopwiki.org/events/851)	GABAergic interneurons, Decreased
8	KE	385	Decrease of synaptogenesis (https://aopwiki.org/events/385)	Synaptogenesis, Decreased

Sequence	Type	Event ID	Title	Short name
9	KE	386	Decrease of neuronal network function (https://aopwiki.org/events/386)	Neuronal network function, Decreased
10	AO	341	Impairment, Learning and memory (https://aopwiki.org/events/341)	Impairment, Learning and memory

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Na ⁺ /I ⁻ symporter (NIS) (https://aopwiki.org/relationships/442)	adjacent	Decrease of Thyroidal iodide	High	High
Decrease of Thyroidal iodide (https://aopwiki.org/relationships/872)	adjacent	Thyroid hormone synthesis, Decreased	High	High
Thyroid hormone synthesis, Decreased (https://aopwiki.org/relationships/305)	adjacent	Thyroxine (T4) in serum, Decreased	High	Moderate
Thyroxine (T4) in serum, Decreased (https://aopwiki.org/relationships/312)	adjacent	Thyroxine (T4) in neuronal tissue, Decreased	Moderate	Low
Thyroxine (T4) in neuronal tissue, Decreased (https://aopwiki.org/relationships/444)	adjacent	Reduced levels of BDNF	Moderate	Low
Reduced levels of BDNF (https://aopwiki.org/relationships/870)	adjacent	Decrease of GABAergic interneurons	Moderate	Low
Decrease of GABAergic interneurons (https://aopwiki.org/relationships/871)	adjacent	Decrease of synaptogenesis	Moderate	Low
Decrease of synaptogenesis (https://aopwiki.org/relationships/358)	adjacent	Decrease of neuronal network function	Low	Low
Decrease of neuronal network function (https://aopwiki.org/relationships/359)	adjacent	Impairment, Learning and memory	High	Low
Inhibition, Na ⁺ /I ⁻ symporter (NIS) (https://aopwiki.org/relationships/1503)	non-adjacent	Impairment, Learning and memory	Moderate	Low
Thyroid hormone synthesis, Decreased (https://aopwiki.org/relationships/1506)	non-adjacent	Impairment, Learning and memory	High	Moderate
Thyroid hormone synthesis, Decreased (https://aopwiki.org/relationships/1504)	non-adjacent	Reduced levels of BDNF	Low	Low
Thyroid hormone synthesis, Decreased (https://aopwiki.org/relationships/1505)	non-adjacent	Decrease of GABAergic interneurons	Low	Low
Reduced levels of BDNF (https://aopwiki.org/relationships/448)	non-adjacent	Decrease of synaptogenesis	Moderate	Low
Reduced levels of BDNF (https://aopwiki.org/relationships/1507)	non-adjacent	Impairment, Learning and memory	Moderate	Moderate

Stressors

Name	Evidence
Perchlorate	High
Nitrate	High
Thiocyanate	High
Dysidenin	Low
Aryltrifluoroborates	Low

Overall Assessment of the AOP

This AOP refers mainly to humans and rodent species (principally rat) with regard to taxa. All the KEs are applicable to either sex ("mixed", as indicated under description of individual KEs and KERs), and the life-stage, for all the KEs, is defined as "during brain development", encompassing foetal and perinatal stage, continuing also during childhood and youth.

Biological Plausibility: The functional relationship between NIS and thyroidal iodide uptake is well established. In the human, NIS mutations are associated with congenital iodide transport defect, a condition characterized by low iodide uptake, hypothyroidism and goiter (Bizhanova and Kopp, 2009; De La Vieja et al., 2000; Pohlenn and Refetoff, 1999). The same is true for the relationship between iodide uptake and serum TH concentration, as it is known that Iodide Deficient (ID) suffer also by low thyroid levels in the blood (Wolff, 1998; DeLange, 2000). The correlation of serum and brain concentrations of TH are supported by a smaller amount of quantitative data but the biological plausibility of this connection is mainly based on the number of studies that show that the brain TH is proportional to the serum TH (Broedel et al., 2003). BDNF is thought to underlie the effects of developmental hypothyroidism but this notion is based mainly on their common physiological role during brain development rather than on solid experimental evidence (Gilbert and Lasley, 2013). On the other hand, the role of BDNF on the GABAergic interneurons development and function is well established, as many experimental data have been produced the last decades in support to this relationship (Woo and Lu, 2006; Palizyan et al., 2004; Patz et al., 2004). It is also widely accepted that the GABAergic signalling and therefore the proper function of GABAergic interneurons is fundamental for the normal synapse formation, which in turn controls the neuronal network formation, maturation and function. Numerous studies have shown that the depolarizing GABA signalling is controlled by the intracellular Cl⁻ concentration in the postsynaptic cells and is the first drive for synapse formation (Wang and Kriegstein, 2008; Cancilla et al., 2007; Ge et al., 2006; Chudotvorova et al., 2005; Akerman and Cline, 2006). This early synaptogenesis period is critical for the establishment of the basic neuronal circuitry, despite the fact that synaptogenesis is a continuous process throughout life (Rodier, 1995). Neonatal hypothyroidism results in altered neuronal structure and function, including reduction in neurite outgrowth, synaptogenesis and dendritic elaborations. RC3/neurogranin is a gene directly regulated by thyroid hormone whose expression is consistent with a role in synapse formation and/or function (Munoz et al., 1991). The specific alterations in dendritic morphology have been identified in several cell types, including pyramidal cells in the cerebral cortex (decrease in dendritic spine number) (Schwartz, 1983), pyramidal cells in the visual cortex (reduced number and altered distribution of dendritic spines) (Morreale de Escobar et al., 1983), cholinergic basal forebrain neurons (decreased number of primary dendrites and number of dendritic branchpoints) (Gould and Butcher, 1989), Purkinje cells (decreased number and size of dendritic spines) (Nicholson and Altman, 1972; Legrand, 1979) and granule and pyramidal cells in the hippocampus (decreased branching of apical and basal dendrites) (Rami et al., 1986). Thus, TH influences the size, packing density and dendritic morphology of neurons throughout the brain, including myelination. Indeed, a striking phenotype in the hypothyroid neonatal brain is the reduction in myelin-protein gene expression (Farsetti et al., 1992; Pombo et al., 1999). However it should be noted that TH role during brain development is complex and still not fully understood.

Dose-response concordance: Multiple events were considered together in only limited number of studies. There is overwhelming evidence that supports the concordance of NIS inhibition with the decrease of thyroidal iodide uptake or the lower levels of serum TH but these two events have rarely been tested together. However, in the few cases that the levels of thyroidal iodide and the serum TH levels are measured in the same study the results are mostly conflicting, mainly due to the well-developed compensatory mechanisms that exist to maintain the TH levels in the body. That means that the effects of NIS inhibitors might not be detectable in short-term or low-dose experiments. Perchlorate is a well-described NIS inhibitor and the interpretation of related studies is straightforward because thyroid is considered the critical effect organ of perchlorate toxicity (National Research Council 2005); thus, any effects of perchlorate on the nervous system are necessarily interpreted to be subsequent to inhibition of iodide uptake by the thyroid gland and to a reduction in serum THs. Indeed, the use of potassium or sodium perchlorate has contributed to the identification of a dose-response relationships between NIS inhibition and thyroidal iodide uptake (Greer et al., 2002; Tonacchera et al., 2004; Cianchetta et al., 2010; Waltz et al., 2010; Lecat-Guillet et al., 2007; 2008) but the respective concordance with serum TH was not shown in most of these studies. On the other hand, in the human and animal studies that revealed a strong dose-dependent association between perchlorate exposure and

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circulating levels of TH (Blount et al., 2006; Cao et al., 2010; Suh et al., 2013; Steinmaus et al., 2007; Steinmaus et al., 2013; Siglin et al., 2000; Caldwell et al., 1995; Argus research laboratories 2001; York et al., 2003; York et al., 2004), the decrease of thyroidal iodide was not investigated. The downstream effects of TH insufficiency are better understood and documented but the majority of the dose-response data are derived from hypothyroid rodents after exposure with propylthiouracil (PTU) and methimazole (MMI), which is the most common used chemicals for the production of hypothyroid state to animals. Those types of experiments give information on the mechanisms through which TH insufficiency leads to neurodevelopmental deficits, but this pathway cannot be connected with NIS inhibition as data on specific NIS inhibitors is still lacking. In regards to the downstream events in the pathway, there is a strong correlation between each KE but the majority of the studies have been performed under severe hypothyroid conditions (high doses of PTU and/or MMI, thyroidectomies); therefore it is difficult to establish the dose-response relationships in each one of them. The association between serum TH levels and BDNF protein in the brain is very well documented but with the exception of few cases (Chakraborty et al., 2012; Blanco et al., 2013) no dose-response experiments are available. The same problem is also encountered in the relationship between BDNF levels and the GABAergic function, as there is only one recent study (Westerholz et al., 2013) that describes a correlation between these two events, but the results are described on the basis of T3 presence or complete absence in the cultures, which does not allow the establishment of dose-response evaluation. However, a dose-response relationship has been shown in earlier studies between the T3 hormone and the density of synapses in cortical cultures, an effect which was paralleled with the electrical activity of the network (Westerholz et al., 2010; Hosoda et al., 2003). More recently, a model of low level TH disruption has been developed, in which different concentrations of PTU have been tested and the subsequent dose-response relationships with GABAergic interneurons expression, synaptogenesis and learning and memory deficits were established (Sui and Gilbert, 2003; Gilbert and Sui, 2006; Gilbert, 2011; Gilbert et al., 2006, 2012; Berbel et al., 1996). Additionally, results from animal studies with perchlorate have also shown a dose-dependent reduction in excitatory and inhibitory synaptic function leading to learning and memory impairments (Gilbert and Sui, 2008). In contrast, there is only limited data in support to the correlation between TH insufficiency and the neuronal network function, and no dose-response relationship can be established.

Temporal concordance: In regards to temporality, the concordance between the KEs from the NIS inhibition until the TH levels in the brain is well-established. It is widely accepted that the most important role of iodine is the formation of the thyroid hormones (T4 and T3) and that iodine deficiency early in development can cause severe hypothyroidism leading to irreversible neurocognitive impairments (DeLange, 2000; Zimmermann et al., 2006). The majority of the data on TH insufficiency is derived from studies performed in different developmental stages and this study design facilitates the establishment of temporal concordance between the downstream KEs in the AOP. In general, TH insufficiency during the prenatal and early post-natal period is correlated with deficits in GABAergic morphology and function, especially of PV-positive interneurons (Berbel et al., 1996; Gilbert et al., 2007; Westerholz et al., 2010; 2013), with the decrease of active synapses and of synchronized electrical activity in cortical networks (Westerholz et al., 2010; Hosoda et al., 2003). This developmental window is known to be critical for the brain development and therefore TH deficits during this period has been correlated with mental retardation and other neurological impairments in children, which in some cases are irreversible (Mirabella et al., 2000; Porterfield and Hendrich, 1993). In at least two studies multiple KEs have been considered together and provide important information on the temporality of the AOP. Westerholz et al., 2010 and 2013 have shown that TH insufficiency during the first two postnatal weeks may cause alterations in the morphology and function of PV-positive GABAergic interneurons, with subsequent effects on the number of active synapses and the electrical activity of the neuronal network. During the same period the inhibition of BDNF function was shown to be also involved in the formation of synaptic connections (Westerholz et al., 2013). Further investigation of the mediating mechanisms revealed that a critical function in the above mentioned cascade was the timely shift of GABA signalling from depolarization to hyperpolarization, a milestone in brain development. The GABA switch takes place at the end of the second postnatal week in rodents, and thus we can conclude that all the KEs are performed during the perinatal period up to 14 days postnatal, which fits in the overall AOP, as this is the critical period for synaptogenesis and subsequently for the proper development of learning and memory functions.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Foetal	High
Perinatal	High
During brain development	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Rattus sp.	Rattus sp.	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10118)

Sex Applicability

Sex	Evidence
Male	High
Female	High

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Essentiality of the Key Events

Key Events	Direct Evidence	Indirect Evidence	No or contradictory experimental evidence

MIE (Na⁺/I⁻ symporter inhibition	<p>Weight of evidence for essentiality of MIE resulting in KE1 <i>Decreased Thyroidal iodine</i> and other KEs downstream is high. A number of studies have demonstrated that cessation of exposure to NIS inhibitors results in a return to normal iodine uptake (e.g. Greer (https://www.ncbi.nlm.nih.gov/pubmed/) et al., 2002, Russet et al., 2015), TH synthesis is recovered and TH levels return to their baseline values. For instance a recovery period of 15-30 days after the exposure to NIS inhibitor (perchlorate) showed that the inhibitory effects were eliminated almost completely, as the measurements of iodide uptake (Greer et al., 2002) and serum TH levels (Siglin et al., 2000) were indistinguishable from their respective baseline values. Also, the use of cells that did not endogenously express the NIS transfer protein prevented completely iodide uptake that was reversed by hNIS transfection (Cianchetta et al., 2010).</p> <p>Three Japanese children inherited two NIS mutations (V59E and T354P) from their healthy mother and father, respectively (Kosugi et al. 1998; Ferrandino et al. 2017). V59E NIS was reported to exhibit as much as 30% of the activity of wild-type NIS (Fujiwara et al. 2000). The T354P and V59E NIS mutant proteins, when expressed in COS7 cells, were both trafficked to the cell surface, but totally inactive. The three siblings displayed different degrees of mental retardation, including heavy learning and memory deficits. The oldest one was nursed for longer than the second oldest, and evinced a less severe cognitive deficit. The youngest was not nursed, and displayed a <u>more severe cognitive deficit than either of her siblings</u>. It was discovered that the mother was addicted to laminaria, an alga extremely rich in I⁻ (Ferrandino et al. 2017).</p>		
KE1 Thyroidal iodine, Decreased	<p>Iodine deficiency is regulated by an addition of iodine to salt and other dietary products. Increased iodine levels in diet compensates decreased TH synthesis and TH levels in blood (Rousset et al., 2015. Dun 1998, 2002; International Council for Control of Iodine Deficiency Disorders. Current Iodine Deficiency Disorders Status Database. http://www.ichidd.org/ (http://www.ichidd.org/).</p> <p>In pregnant women mild hypothyroxinemia due to iodine deficiency leads to altered neurocognitive performance (AO of this AOP) of the progeny. This hypothyroxinemia was corrected with iodine supplements during the first trimester (La Gamma et al., 2006).</p> <p>In vitro study using thyroid follicular FRTL-5 cells, showed that incubation with hydrogen peroxide decreased NIS-mediated I⁻ transport, and this effect was reverted by adding ROS scavengers (Arriagada et al., 2015).</p>		
KE2 Thyroid hormone synthesis, Decreased	<p>Several studies have proven that NIS inhibitors lead to a decrease of thyroidal iodide uptake resulting in a reduction of TH synthesis (e.g. Jones et al., 1996; Tonacchera et al., 2004; De Groef et al., 2006; Waltz et al., 2010). Removing exposure to NIS inhibitors reverses decreased TH synthesis (as described above). Similar studies are published for decreased TH synthesis induced by TPO inhibitors.</p>		
KE3 T4 in serum, Decreased	<p>There is strong evidence that decreased Thyroxine (T4) synthesis in the thyroid gland results in decreased T4 concentration in serum ((Dong et al., 2017; Calil-Silveira et al., 2016; Tang et al., 2013; Liu et al., 2012; Pearce et al., 2012). Recovery experiments (cessation an exposure to NIS or TPO inhibitors) demonstrate recovery of serum T4 concentrations (Dong et al., 2017; Calil-Silveira et al., 2016; Tang et al., 2013; Liu et al., 2012; Pearce et al., 2012; Steinmaus, 2016a, 2016b; Wu Y et al., 2016).</p> <p>T4 or T3 treatment during critical developmental windows, was shown to restore (or reduce) structural alterations in brain (Goodman and Gilbert, 2007; Auso et al., 2004; Lavado-Autric et al., 2003; Berbel et al., 2010; Koiuchi and Chin, 2000). For instance, Auso et al., 2004 showed that infusion of dams with T4 <u>between E13 and E15</u> prevented alterations of the cytoarchitecture and the radial distribution of BrdU+ neurons in the somatosensory cortex and hippocampus (Ausso et al., 2004).</p> <p>T3 or T4 were administered to wild-type (WT) and to Mct8KO mice previously made hypothyroid. The Mct8KO mice only responded to T4 which reached the brain in the Mct8-deficient mice through Oatp1c1 transporter. D2 activity was responsible for normal expression of most brain TH-regulated functions that was compromised in the absence of Mct8 (Morte et al., 2010; Bernal, 2015).</p> <p>Calvo et al. (1990) showed that T4 and T3 administration restored both serum and tissue levels of TH in gestating hypothyroid rats.</p> <p>Vara et al., 2002 showed that T3 administration in hypothyroid rats <u>recovered neuronal network function</u>, as shown by analysis of Ca(2+)-dependent neurotransmitter release.</p> <p>Sawano et al., 2013 showed that GAD65 protein (GABAergic marker) was reduced by more than 50% of control in the hippocampus of hypothyroid rats, but daily T4 replacement after birth <u>recovered GAD65 protein to control levels</u>.</p> <p>In humans, hormone insufficiency that occurs in mid-pregnancy due to maternal drops in serum hormone, and that which occurs in late pregnancy due to disruptions in the fetal thyroid gland lead to different patterns of cognitive impairment (Zoeller and Rovet, 2004). In animal models, deficits in hippocampal-dependent cognitive tasks result from developmental, but not adult hormone deprivation (Gilbert and Sui, 2006; Gilbert et al., 2016; Axelstad et al., 2009; Gilbert, 2011; Opazo et al., 2008). Replacement studies have demonstrated that varying adverse neurobehavioral outcomes, including learning and memory impairment, can be reduced or eliminated if T4 (and/or T3) treatment is given during the critical windows (e.g., Kawada et al., 1988; Reid et al., 2007).</p>	<p>While T4 and T3 administration restored both serum and tissue levels of TH in gestating hypothyroid rats, recovery of TH levels (in serum and tissues) occurred only partially in fetal tissues (Calvo et al., 1990).</p> <p>Wang et al., 2012 have shown that L-T4 treatment (at GD10 and GD13) <u>ameliorated</u> the adverse effect of maternal subclinical hypothyroidism on <u>spatial learning and memory (AO) in the offspring</u>.</p> <p>Wang et al., 2012 also showed that T4 treatment <u>ameliorated BDNF expression changes</u> in the progeny of rats with subclinical hypothyroidism.</p> <p>Pathak et al. 2011 showed that TH administration (at E13-15 in MMI-treated rat dams) <u>recovered</u> the number and length of radial glia, the loss of neuronal bipolarity, and the impaired neuronal migration (<u>indicative of decreased synaptogenesis</u>) observed in hypothyroid offspring.</p> <p>Di Liegro et al. (1995) showed that in primary cultures T3 treatment induces the expression of synapsin I (increased synaptogenesis).</p>	<p>Infusion of dams with T4 <u>after E18 did not prevent</u> alterations of somatosensory cortex and hippocampus cytoarchitecture (Ausso et al., 2004).</p> <p>Wang et al., 2012 also showed that T4 treatment <u>at GD17 had only minimal effects on spatial learning in the offspring</u>.</p> <p>Gilbert et al., 2007 showed that PV+ cells (GABAergic) were diminished in the hippocampus and neocortex of hypothyroid offspring. <u>Return of TH to control levels in adulthood was not associated with higher PV+ cell numbers</u>.</p>

KE4 T4 in neuronal tissue Decreased	<p>Several studies have demonstrated that fetal brain TH levels, previously decreased by maternal exposure to TH synthesis inhibitors or thyroideectomy, recovered following maternal supply of T4 (e.g., Calvo et al., 1990). However, there are no studies with direct infusion of T4 or T3 directly into brain.</p>	<p>The upregulation of deiodinase has been shown to compensate for some loss of neuronal T3 (Escobar-Morreale et al., 1995; 1997).</p> <p>Indirect evidence shows that T4 replacement that brings circulating T4 concentration back to physiological levels normal, leads to recovery of brain TH and prevents downstream effects including alterations in cell morphology, differentiation and function.</p>	
KE5 BDNF release, Reduced	<p>It is well known fact that BDNF is critical for neuronal differentiation and maturation, including synaptic integrity and neuronal plasticity in hippocampus and cortex, two brain structures that are essential for learning and memory processes in animals and humans. Limited data from studies in BDNF knockout animals demonstrate that deficits in hippocampal synaptic transmission and plasticity, and downstream key events can be rescued with recombinant BDNF (Aarse et al., 2016; Andero et al., 2014). A few examples are briefly described below.</p> <p>In <i>in vivo</i> studies on hypothyroid rat models, exposed to TPO inhibitors (MMI, PTU), and/or NIS inhibitor (perchlorate) offspring showed reductions in BDNF mRNA and protein levels, and the most affected brain regions were two brain structures critical for learning and memory processes, such as hippocampus and cortex, and the cerebellum (Koibuchi et al., 1999; 2001; Sinha et al., 2009; Neveu and Arenas, 1996; Gilbert and Lasley, 2013). Following a T4 dosing regimen in rats an increased BDNF mRNA and protein expression was observed (e.g. Camboni et al., 2003; (http://www.sciencedirect.com/science/article/pii/S0306452212011335#b0120); Lüesse et al., 1998 (http://www.sciencedirect.com/science/article/pii/S0306452212011335#b0450)).</p> <p>Inhibition of BDNF by K252a (a Trk antagonist) in cultures containing T3 resulted in decreased number of synaptic boutons, (critical for synaptogenesis) as in the T3-deprived cultures (Westerholz et al., 2013). T3-deficient rat cultures of cortical PV⁺ GABA interneurons, found that the number of synaptic boutons was reduced but exogenous BDNF application abolished this effect (Westerholz et al., (2013)).</p> <p>Limited data from studies in BDNF knockout animals demonstrate that deficits in hippocampal synaptic transmission and plasticity, and downstream behaviors can be rescued with recombinant BDNF (Aarse et al., 2016; Andero et al., 2014).</p> <p>Aguado et al., 2003 showed that BDNF <u>overexpression</u> in transgenic embryos increased the number of synapses (<u>increased synaptogenesis</u>), and increased spontaneous neuronal activity (<u>increased neuronal network function</u>), and <u>increased the number of GABAergic interneurons</u>, indicating that BDNF is essential to control both GABAergic pre- and postsynaptic sites.</p> <p>Neveu and Arenas, 1996 found that early hypothyroidism (by PTU administration to rat dams) decreased the expression of neurotrophin 3 (NT-3) and BDNF mRNA. Grafting of P3 hypothyroid rats with cell lines overexpressing BDNF (or NT-3) <u>prevented hypothyroidism-induced cell death</u> in neurons of the internal granule cell layer at P15.</p> <p>BDNF application elicits presynaptic changes in GABAergic interneurons, as several presynaptic proteins were up-regulated after BDNF application (Yamada et al., 2002; Berghuis et al., 2004). Increase of GABA receptor density was observed in cultured hippocampus-derived neurons after treatment with BDNF (Yamada et al., 2002).</p> <p>Westerholz et al., (2013), by using rat T3-deficient cultures of cortical PV⁺ interneurons, found that the number of synaptic boutons (critical for synaptogenesis) was reduced but exogenous BDNF application abolished this effect.</p> <p>In this <i>in vivo</i> study, the protein synthesis inhibitor anisomycin (Ani; 80 µg/0.8 µl per side) was injected in the dorsal hippocampus of Male Wistar rats (2.5 months) 12 h after inhibitory avoidance (IA) training (i.e., using a strong foot shock, which generates a persistent LTM), which causes a selective deficit in memory retention 7 days, but not 2 days, after training. Human recombinant BDNF (rhBDNF, 0.25 µg/0.8 µl per side) or vehicle (Veh) was delivered 15 min after Ani infusion into the hippocampus. <u>rhBDNF completely rescued long-term memories (LTM)</u> at 7 days after training caused by Ani given at 12 h after training. Additionally, infusion of <u>BDNF antisense oligonucleotides</u> (i.e., BDNF ASO, which blocks the expression of BDNF 12 h after training) into the dorsal hippocampus 10 h after training, was found to impair persistence (a characteristic feature of LTM), but not formation of IA LTM (as compared with BDNF missense oligonucleotide). This indicates that <u>BDNF during the late posttraining critical time period is not only required but sufficient for persistence of LTM storage</u> (Bekinschtein et al. 2008).</p> <p>In this study the role of BDNF in both short and long term memories (STM and LTM) formation of a hippocampal-dependent one-trial fear-motivated learning task was examined in male Wistar rats (2–3 months). IA training was found associated with a rapid and transient increase in BDNF mRNA expression (by 90%, 1 hr after IA training) in the hippocampus. Bilateral infusions of function-blocking <u>anti-BDNF antibody</u> (0.5 µg/side) into the CA1 region of the dorsal hippocampus decreased ERK2 activation, and blocked STM formation. On the contrary, intrahippocampal administration of rhBDNF (0.25 µg/side) increased ERK1/2 activation and facilitated STM. These results strongly indicate that endogenous BDNF is required for both STM and LTM formation of an IA learning (Alonso et al. 2002).</p>	<p>Beta-estradiol (E2) induced synaptogenesis by enhancing BDNF release from dentate gyrus (DG) granule cells measured by increased the expression of PSD95, a postsynaptic marker. E2 effects were completely inhibited by blocking the BDNF receptor (TrkB) with K252a or by using a function-blocking antibody to BDNF, which inhibited the expression of PSD95. Both K252a and the antibody anti-BDNF elicited a decrease of spine density (presynaptic sites) (Sato et al., 2007).</p> <p>Intrahippocampal microinfusion of BDNF modulated the ability of the hippocampal mossy fiber pathway to produce long-term potentiation (LTP) by high frequency stimulation. On the opposite, administration of the TrkB inhibitor K252a, in combination with BDNF, blocked the functional and morphological effects produced by BDNF (Schjetnan and Escobar, 2012). These data confirm the role of BDNF in the regulation of synaptic plasticity and <i>Neuronal Network Function</i> (KE downstream)</p> <p>In heterozygous BDNF knockout (BDNF^{+/−}) mice, a decrease of NMDAR-independent mossy fiber LTP occurred. Inhibition of TrkB/BDNF signalling with K252a, or with the selective BDNF scavenger TrkB-Fc inhibited mossy fiber LTP to the same extent as observed in BDNF^{+/−} mice (Schildt et al., (2013)), supporting an important role of BDNF in <i>Neuronal Network Function</i> (KE downstream).</p> <p>Exogenous application of BDNF in developing neocortical and hippocampal GABAergic interneurons has demonstrated an <u>enhanced dendritic elongation and branching in cultures (synaptogenesis)</u> (Jin et al., 2003; Vicario-Abejon et al., 1998; Marty et al., 2000).</p> <p>Endogenous BDNF promotes interneuron differentiation (Kohara et al., 2003).</p>	

KE6 GABAergic interneurons, Decreased	<p>There are limited studies in support of this KE.</p> <p>Prenatal exposure to TPO inhibitors (PTU or MMI, to induce hypothyroidism), decreased number of the GABAergic interneurons (parvalbumin (PV)⁺ cells) and glutamic acid decarboxylase 65 (GAD65)⁺ cells (e.g. Sawano et al., 2013; Shiraki et al., 2012; Gilbert et al., 2007).</p> <p>Bisphenol-A (BPA), inhibitor of NIS (Wu Y et al., 2016) decreased KCC2 mRNA expression and attenuated [Cl⁻]_i shift in migrating cortical inhibitory precursor neurons, as observed in primary rat and human cortical neurons (Yeo et al., 2013).</p>	<p>Transcriptional repression of KCC2 (responsible for neuronal Cl⁻ homeostasis) delays the GABAergic switch (Yeo et al., 2009). The absence of T3 in cultures of cortical GABAergic interneurons also delays the developmental KCC2 up-regulation and subsequently the GABA shift, with a profound decrease in the number of synapses (Westerholz et al., 2010; 2013), proving that early synaptogenesis network activity is under control of TH mediated through GABAergic interneurons.</p>	
KE7 Synaptogenesis, Decreased	<p>The connectivity and functionality of neural networks depends on where and when synapses are formed (synaptogenesis). Therefore, the decreased synapse formation during the process of synaptogenesis is detrimental and leads to decrease of neural network formation and function. The neuronal electrical activity dependence on synapse formation and is critical for proper neuronal communication. Alterations in synaptic connectivity lead to refinement of neuronal networks during development (Cline and Haas, 2008). It is well established fact that hypothyroidism decreases synaptogenesis resulting in synaptic transmission and plasticity impairments (Vara et al., 2002; Sui and Gilbert, 2003; Gilbert, 2004; Dong et al., 2005; Sui et al., 2005; Gilbert and Paczkowski, 2003; Gilbert and Sui, 2006; Gilbert, 2011; Gilbert et al., 2013). Indeed, pyramidal neurons of hypothyroid animals have fewer synapses and an impoverished dendritic arbor (Rami et al., 1986; Madeira et al., 1992). It has been demonstrated that the decreased expression of genes critical for synaptogenesis (e.g. Srg1, RC3/neurogranin, a Hairless Homolog) in hypothyroid rats can be reversed by an administration of TH (Thompson, 1996; Potter et al., 2001; Thompson and Potter, 2000). For example Srg1 (Synaptotagmin-related gene 1) mRNA expression is reduced ~3-fold in rat hypothyroid cerebellum. Injection of thyroid hormone causes a very rapid induction of Srg1 (in 2 hrs) (Thompson, 1996; Potter et al., 2001) suggesting that this gene is a direct target of thyroid hormone action.</p> <p>In mutant mice lacking PSD-95, it has been recorded increase of NMDA-dependent LTP, at different frequencies of synaptic stimulation that cause severe impaired spatial learning, without thought affecting the synaptic NMDA receptor currents, subunit expression, localization and synaptic morphology (Migaud et al., 1998). Furthermore, recent genetic screening in human subjects and neurobehavioural studies in transgenic mice have suggested that loss of synaptophysin plays important role in mental retardation and/or learning deficits (Schmitt et al., 2009; Tarpey et al., 2009).</p>		
KE8 Neuronal Network Function, Decreased	<p>It is well understood and documented that the ability of neurons to communicate with each other is based on neural network formation that relies on functional synapse establishment (Colón-Ramos, 2009). Indeed, decreased neuronal network function in developing brain (dysfunction of synaptic connectivity, transmission and plasticity) contribute to the impairment of learning and memory. A number of studies 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 within neuronal networks 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). It is well documented that hippocampal regions (i.e., area CA1 and dentate gyrus) exhibit alterations in network function of 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 deficits persist into adulthood, long after recovery to euthyroid status, suggesting that they might be only partially reversible.</p>		
AO Learning and memory, Impairment	<p>The essentiality of the relationship between decreased TH levels and learning and memory deficit is well-documented based on the existing literature. TH plays a critical role for normal nervous system development and function, including learning and memory processes (e.g. Williams, 2008; Bernal, 2015). This includes particularly development of the hippocampus and cortex, two brain regions that play a major role in spatial, temporal, and contextual memory. Indeed, most developed countries check for childhood hypothyroidism at birth to immediately begin replacement therapy. This has been shown to alleviate most adverse impacts of hypothyroidism in congenitally hypothyroid children (Derksen-Lubsen and Verkerk 1996; Zoeller and Rovet, 2004). Similar results are also produced in animal studies showing that T4 treatment reverses spatial learning deficits induced by maternal hypothyroidism in rats (e.g. Wang et al., 2012). In the context of NIS inhibitors Taylor and co-workers found that levels of urinary perchlorate assessed in a cohort study of 21,846 women, were positively associated with a higher risk for children having lower IQ scores at 3 years of age (Taylor et al., 2014).</p>		

Quantitative Consideration

Some semi-quantitative data are available for the described KERs; however, further experimental work is needed to define thresholds suitable to assess when a given KE-downstream will be triggered by the KE-upstream.

Considerations for Potential Applications of the AOP (optional)

The US EPA and OECD Developmental Neurotoxicity (DNT) Test Guidelines (OCSPP 870.6300 and OECD 426, respectively) require testing of learning and memory. These DNT guidelines are based entirely on *in vivo* experiments, which are costly, time consuming, and unsuitable for testing a larger number of chemicals. At the same time the published data strongly suggest that environmental chemicals contribute to the recent observed increase of neurodevelopmental disorders in children such as lowered IQ, learning disabilities, attention deficit hyperactivity disorder (ADHD) and, in particular, autism. This highlights the pressing need for standardised alternative methodologies that can more rapidly and cost-effectively screen large numbers of chemicals for their potential to cause cognitive deficit in children.

This AOP can encourage the development of new *in vitro* test battery anchored to the KEs identified in the AOP. The majority of KEs in this AOP has strong essentiality to induce the AO (impairment of learning and memory) and established indirect relationship with the AO that would allow not only the development of testing methods that address these specific KEs but also the understanding of the relationship between the measured KEs and the AO.

Therefore, this AOP can potentially provide the basis for development of a mechanistically informed Integrated Approaches and Testing Assessment (IATA) to identify chemicals with potential to cause impairment of learning and memory. It should be noted that it is not necessary to quantify all the intermediate KEs defined in an AOP pathway to enable computational modelling to proceed to a quantitative model that would predict cognitive outcomes from *in vitro* data.

This AOP stimulated recent efforts to develop screening radioactive (Dohan et al., 2003; Riesco-Eizaguirre and Santisteban, 2006) and non-radioactive (Waltz et al., 210) *in vitro* assays to detect potential NIS inhibitors that could inform quantitative structure activity relationships, read-across models, and/or systems biology models to prioritize chemicals for further testing (Waltz et al., 2010).

Finally, this AOP could provide the opportunity to group chemicals based not only on their physical- chemical properties but also their biological activity (biological grouping) referring to the triggered key events.

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Appendix 1

List of MIEs in this AOP

Event: 424: Inhibition, Na⁺/I⁻ symporter (NIS) (<https://aopwiki.org/events/424>)

Short Name: Inhibition, Na⁺/I⁻ symporter (NIS)

Key Event Component

Process	Object	Action
sodium:iodide symporter activity	sodium/iodide cotransporter	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/65)	MolecularInitiatingEvent
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	MolecularInitiatingEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	MolecularInitiatingEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/176)	MolecularInitiatingEvent

Stressors

Name
Perchlorate
Nitrate

Name
Thiocyanate
Dysidenin
Aryltrifluoroborates
Econazole
5-(N,N-hexamethylene) amiloride (HMA)
Small molecules: ITB3, ITB4, ITB5, ITB9

Biological Context

Level of Biological Organization
Molecular
Cell term
Cell term
thyroid follicular cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Thyroid Disrupting Chemicals (TDCs) are defined as the xenobiotics that interfere with the thyroid axis with different outcomes for the organism. A very well-studied mechanism of action of the TDCs is the reduction of the circulating levels of THs by inhibiting hormone synthesis in the thyroid gland. For example, perchlorate is a very potent inhibitor of iodide uptake through the sodium/iodide symporter (Tonacchera et al., 2004). Perchlorate has been detected in human breast milk ranging from 1.4 to 92.2 mg μ l⁻¹ (10.5 μ g l⁻¹ mean) in 18 US states (Kirk et al. 2005), and 1.3 to 411 μ g l⁻¹ (9.1 μ g l⁻¹ median) in the Boston area, United States (Pearce et al. 2007). Perchlorate has also been detected in human colostrum of 46 women in the Boston area (from < 0.05 to 187.2 μ mol l⁻¹ (Leung et al. 2009)). The mechanism of perchlorate action is quite simple, as it is believed to be mediated only by the NIS inhibition (Dohan et al., 2007; Wolff, 1998). Additionally, thiocyanate and nitrate are two known inhibitors that have been found to reduce circulating TH levels (Blount et al., 2006; Steinhaus et al., 2007), but they are both less potent than perchlorate (Tonacchera et al., 2004). However, there are also contradictory results from other studies that showed no correlation between thyroid parameters and perchlorate levels in humans (Pearce et al., 2010; Amitai et al., 2007; Tellez et al., 2005).

Co-occurrence of perchlorate, nitrate, and thiocyanate can alter thyroid function in pregnant women. Horton et al. (2015) have shown positive associations between the weighted sum of urinary concentrations of these three analytes and increased TSH, with perchlorate showing the largest weight in the index. Interestingly, De Groef et al. 2006 showed that nitrate and thiocyanate, acquired through drinking water or food, account for a much larger proportion of iodine uptake inhibition than perchlorate, suggesting that NIS inhibition and any potential downstream effect by perchlorate are highly dependent on the presence of other environmental NIS inhibitors and iodine intake itself (Leung et al., 2010). In particular, Tonacchera et al. (2004) showed that the relative potency of perchlorate to inhibit radioactive I⁻ uptake by NIS is 15, 30 and 240 times that of thiocyanate, iodide, and nitrate respectively on a molar concentration basis. These data are in line with earlier studies in rats (Alexander and Wolff, 1996; Greer et al. 1966). Contradictory findings in these studies may therefore be a result of the confounding mixtures in the environment, masking the primary effect of perchlorate.

Decreased iodine intake can decrease TH production, and therefore exposure to perchlorate might be particularly detrimental in iodine-deficient individuals (Leung et al. 2010). Moreover, biologically based dose-response modeling of the relationships among iodide status (e.g., dietary iodine levels), perchlorate dose, and TH production in pregnant women has shown that iodine intake has a profound effect on the likelihood that exposure to goitrogens will produce hypothyroxinemia (Lewandowski et al. 2015).

During pregnancy TH requirements increase, particularly during the first trimester (Alexander et al. 2004; Leung et al. 2010), due to higher concentrations of thyroxine-binding globulin, placental T4 inner-ring deiodination leading to the inactive reverse T3 (rT3), and transfer of small amounts of T4 to the foetus (during the first trimester foetal thyroid function is absent). Moreover, glomerular filtration rate and clearance of proteins and other molecules are both increased during pregnancy, possibly causing increased renal iodide clearance and a decreased of circulating plasma iodine (Glienoer, 1997). Thus, even though the foetal thyroid can trap iodide by about 12 week of gestation (Fisher and Klein, 1981), high concentrations of maternal perchlorate may potentially decrease thyroidal iodine available to the foetus by inhibiting placental NIS (Leung et al. 2010).

Consequences of TH deficiency depend on the developmental timing of the deficiency (Zoeller and Rovet, 2004). For instance, if the TH deficiency occurs during early pregnancy, offspring show visual attention, visual processing and gross motor skills deficits, while if it occurs later, offspring may show subnormal visual and visuospatial skills, along with slower response speeds and motor deficits. If TH insufficiency occurs after birth, language and memory skills are most predominantly affected (Zoeller and Rovet, 2004).

Along this line, age and developmental stage are crucial in determining sensitivity to NIS inhibitors (e.g., perchlorate, thiocyanate, and nitrate). In this regard, McMullen et al. (2017) have shown that adolescent boys and girls, more than adults, represent vulnerable subpopulations to NIS symporter inhibitors. Altogether these studies indicate that age, gender, developmental stage, and dietary iodine levels can affect the impact of NIS inhibitors.

Finally, ten more small simple-structured molecules were identified in a large screening study (Lecat-Guillett et al., 2008b) that could block iodide uptake by specifically disrupting NIS in a dose-dependent manner. These molecules were named Iodide Transport Blockers (ITBs). There are few organic molecules that lead to NIS inhibition but no direct interaction with NIS has been determined (Gerard et al., 1994; Kaminsky et al., 1991). Up to date, only dysidenin, a toxin isolated from the marine sponge *Dysidea herbacea*, has been reported to specifically inhibit NIS (Van Sande et al., 2003). Finally, the aryltrifluoroborates were found to inhibit iodide uptake with an IC50 value of 0.4 μ M on rat-derived thyroid cells (Lecat-Guillett et al., 2008a). The biological activity is rationalized by the presence of the BF3- ion as a minimal binding motif for substrate recognition at the iodide binding site.

It has been also shown that many anions, such as ClO₄⁻, SCN⁻, NO₃⁻, ReO₄⁻, TcO₄⁻ and in a lower extent Br⁻ and BF4⁻, are also acting as NIS substrates and they enter the cell by the same transporter mechanism (Van Sande et al., 2003). It has been also shown that ClO₄⁻ is transferred by NIS with high affinity and is considered as one of its most potent inhibitors (Dohan et al., 2007). Most recently, the aryltrifluoroborates were also shown to inhibit NIS function (Lecat-Guillett et al., 2008a). A library of 17,020 compounds was tested by a radioactive screening method with high specificity using transfected mammalian cells (Lecat-Guillett et al., 2008b; 2007) for NIS inhibition evaluation. Further studies with the most powerful inhibitors showed a high diversity in their structure and mode of action (Lindenthal et al., 2009).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	<i>Mus musculus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Pig	Pig	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
zebra fish	<i>Danio rerio</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
Xenopus (Silurana) epitropicalis	<i>Xenopus (Silurana) epitropicalis</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=224340)

Life Stage Applicability

Life Stage	Evidence
Pregnancy	High
Birth to < 1 month	High
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

Apart from the human, functional NIS protein has been also identified in different species, including the rat (Dai et al., 1996), the mouse (Perron et al., 2001), the pig (Selmi-Ruby et al., 2003), zebrafish (Thienpont et al., 2011) and in xenopus (amphibian) (Lindenthal et al., 2009). Mouse and rat contain 618 amino acid residues, while the human and pig contain 643. There are several NIS variants that produce three active proteins in the pig due to alternative splicing at mRNA sites that are not present on the other species (Selmi-Ruby et al., 2003).

NIS orthologs are discussed in the review by Darrouzet's group (Darrouzet et al., 2014). Interestingly, functional differences have been identified between mouse or rat NIS (mNIS or rNIS, respectively) and human NIS (hNIS). The rat and the mouse orthologs were shown to accumulate radioisotopes more efficiently than the human protein (Dayem et al., 2008; Heitemes et al., 2003). The molecular basis of these functional differences could be helpful for further characterization of NIS. Zhang and collaborators showed that rNIS is localized in a higher proportion at the plasma membrane than hNIS and the N-terminal region up to putative TM7 appears to be involved in this difference (Zhang et al., 2005). These authors also reported differences in the kinetics of the Na⁺ binding, implicating the region spanning from TM4 to TM6 and Ser200 of hNIS. They, thus, proposed that this region could be involved in sodium binding (Zhang et al., 2005). In our laboratory, it was shown that the Vmax of the mouse protein is four times higher than the Vmax of the human protein when expressed in the same cell line (HEK-293) (Dayem et al., 2008; Darrouzet et al., 2014). The Km_{Na} value determined for hNIS (9.0 ± 0.8 μM) was significantly lower than the Km_{Na} for the mouse protein (26.4 ± 3.5 μM) whereas the Km_{Na} values were not significantly different. Similarly to the rat protein, mNIS is predominantly localized in the plasma membrane whereas the human ortholog is detected intracellularly in 40% of the cells in which it is expressed (Darrouzet et al., 2014). However, the difference in the Vmax values does not only seem to be related to the higher intracellular localization of hNIS. Using chimeric proteins between human and mouse NIS, we showed that the N-terminal region up to TM8 is most probably involved in iodide binding, and that the region from TM5 to the C terminus could play an important role in targeting the protein to the plasma membrane (Dayem et al., 2008). One of the long-term goals of these studies is the engineering of a chimeric NIS protein most suitable for gene therapy, i.e. preserving regions responsible for the high turnover rate and the efficient plasma membrane localization of the mouse protein while replacing the immunogenic extracellular regions with those of the human ortholog. The porcine NIS gene gives rise to splice variants leading to three active NIS proteins with differences in their C-terminal extremities [4]. However, it is not known if these differences lead to distinct properties (Darrouzet et al., 2014).

There is evidence that the MIE (NIS inhibition) is of relevance also for fish as an expression of the slc5a5 transcript (sodium/iodide co-transporter) has been described by various publications for the zebrafish embryo (see www.zfin.org (<http://www.zfin.org>)). It has been demonstrated that NIS inhibitors in zebra fish lead also to a strong repression of thyroid hormone levels (Thienpont et al., 2011) and in xenopus (amphibian) to inhibition of the iodide-induced current (Lindenthal et al., 2009).

Key Event Description

Biological state: Sodium/Iodide symporter (NIS) is a key protein in the thyroid function and its role has been thoroughly investigated after the determination of its molecular identity a few decades ago (Dai et al., 1996). NIS is an intrinsic membrane glycoprotein and it belongs to the superfamily of sodium /solute symporters (SSS) and to the family of human transporters SLC5 (De La Vieja, 2000; Jung, 2002). Its molecular weight is 87 kDa and it contains 13 transmembrane domains that transport 2 sodium cations (Na⁺) for each iodide anion (I⁻) into the follicular thyroid cell (Dohan et al., 2003). The regulation of NIS protein function is usually cell- and tissue-specific (Hingorani et al., 2010) and it is done at the transcriptional and posttranslational levels, including epigenetic regulation (Darrouzet et al., 2014; Russo et al., 2011a). One of the major NIS regulators is the thyroid stimulating hormone (TSH), which has been shown to enhance NIS mRNA and protein expression, therefore it can contribute to restore and maintain iodide uptake activity (Saito et al., 1997; Kogai et al., 2000). At the posttranslational level TSH also contributes to NIS regulation but the specific mechanisms that underlie these effects are still under investigation (Riedel et al., 2001).

Biological compartments: NIS protein is mainly found at the basolateral plasma membrane of the thyroid follicular cells (Dai et al., 1996), where it actively mediates the accumulation of iodide that is the main component of thyroid hormone synthesis and therefore is considered as a major regulator of thyroid homeostasis. NIS also mediates active I⁻ transport in extrathyroidal tissues but it is commonly agreed that is regulated and processed differently in each tissue. Functional NIS protein has been found in salivary gland ductal cells (Jhiang et al., 1998; La Perle et al., 2013), in the mammary gland during lactation (Perron et al., 2001; Cho et al., 2000), lung epithelial cells (Fragoso et al., 2004), intestinal enterocytes (Nicola et al., 2009), stomach cells (Kotani et al., 1998), placenta (Bidart et al., 2000) and testicular cells (Russo et al. 2011b). Additionally, contradictory results have been obtained regarding the NIS expression in human kidney tissue (Lacroix et al., 2001; Spitzweg et al., 2001). In the case of the lactating breast, it is suggested that NIS serves the transfer of iodide in the cells and it subsequent accumulation in the milk, thereby supplying newborns with this component during this sensitive developmental period (Tazebay et al., 2000). Additionally, NIS mRNA has been detected in various other tissues, such as colon, ovaries, uterus, and spleen (Perron et al., 2001; Spitzweg et al., 1998; Vayre et al., 1999), but the functional NIS protein and the site of its localization has not been verified.

General role in biology: The NIS is known in the field of thyroidology because of its ability to mediate the active transport of I⁻ into the thyrocytes, which is the first and most crucial step for T3 and T4 biosynthesis (Dohan et al., 2000). NIS is located on the basolateral membrane of the thyrocytes and co-transports 2 sodium ions along with 1 iodide (2:1 stoichiometry). The electrochemical gradient of sodium serves as the driving force for iodide uptake and it is generated and maintained by the Na⁺/K⁺-ATPase pump, which is located in the same membrane of the thyrocytes. The iodide molecules, after their active transport in the cytoplasm, are passively translocated in the follicular lumen via the transporter protein pendrin and possibly other unknown efflux proteins that are located on the apical membrane (Bzhanova and Kopp, 2009). Subsequently, the thyroid hormones are synthesized in the follicular lumen by incorporating the accumulated iodide, a process which is significantly suppressed in case of NIS dysfunction or inhibition (reviewed in Spitzweg and Morris, 2010). NIS is the last thyroid-related component to be expressed during development at the 10th gestational week, which temporally coincides with the onset of thyroid function and hormonogenesis (Szinnai et al., 2007). Albeit the localization of NIS is not fully completed at this stage, the iodide accumulation has already started. Mutations of NIS gene (SLC5A5) cause expression of non-functional NIS molecule leading to inability of the thyrocyte to accumulate iodide (Matsuda and Koshugi, 1997; Pohlenz et al., 1998), a condition called iodide transport defect (ITD). This is a rare autosomic recessive disease, which if not properly treated is clinically identified by congenital hypothyroidism, goiter, low I⁻ uptake, low saliva/plasma I⁻ ratio and mental impairment of varying degrees (Dohan et al., 2003). Up to date 13 mutations have been described in the NIS gene (Spitzweg and Morris, 2010) and each one of them produces mutants with different structure but in all cases non-functional. The extensive study after NIS molecular characterization and the numerous findings have convinced the scientists that is one of the most crucial components of the entire thyroid system. Additionally, after the realization that NIS could be also used as diagnostic and therapeutic tool for thyroid and non-thyroid cancers (Portulano et al., 2013) a new research activity concerning this specific mechanism has been initiated.

How it is Measured or Detected

There are several methods that are used nowadays to detect the functionality of NIS but none of these methods is OECD validated (OECD Scoping document, 2017). The most well established methods are the following:

1. Measurement of radioiodide uptake (¹²⁵I) in NIS expressing cells. For this method the FRTL5 cell line is the most commonly used, as it endogenously express the NIS protein, but also NIS transfected cell lines have been successfully implemented in many cases (Lecat-Guillet et al., 2007; 2008b; Lindenthal et al., 2009). Once inhibitory activity is identified for a compound then further tests are performed in order to verify that the observed effect is specific due to NIS inhibition. This method has been also adapted in a high throughput format and has been already used for the screening of a chemical library of 17,020 compounds (Lecat-Guillet et al., 2008b).
2. More recently a non-radioactive method has been developed, which has been also adapted in a high throughput format (Waltz et al., 2010). It is a simple spectrophotometric assay for the determination of iodide uptake using rat thyroid-derived cells (FRTL5) based on the catalytic effect of iodide on the reduction of yellow cerium(IV) to colorless cerium(III) in the presence of arsenious acid (Sandell-Kolthoff reaction). The assay is fast, highly reproducible and equally sensitive with the radioiodine detection method.
3. A fluorescence-based method has been developed, which uses the variant YFP-H148Q/I152L of the Yellow Fluorescent Protein (YFP) in order to detect the efflux of iodide into the rat FRTL5 cells. As a positive control perchlorate is used as it is a well known competitive inhibitor of iodide transport by NIS. Fluorescence of recombinant YFP-H148Q/I152L is suppressed by perchlorate and iodide with similar affinities. Fluorescence changes in FRTL-5 cells are Na⁺-dependent, consistent with the Na⁺-dependence of NIS activity. It is supposed to be an innovative approach to detect the cellular uptake of perchlorate and characterize the kinetics of transport by NIS. This method needs further optimization, as YFP is not specific for iodide and thus binding of other ionic molecules could affect the results of the assay (Cianchetta et al., 2010; Rhoden et al., 2008; Di Bernarde et al., 2011).
4. In vivo ¹²⁵I uptake assays is based on immunofluorescence analyses of thyroid glands after the treatment of rat with excess I⁻, injected with Ci Na¹²⁵I as previously described by Ferreira et al., 2005. Then the thyroid glands are removed and weighed, and the amount of ¹²⁵I in the thyroid gland is measured in a γ-counter (PerkinElmer; model Wizard). The counts per minute in the thyroid gland are used to calculate the percentage of ¹²⁵I in the thyroid gland, having in account that 100% corresponded to the counts per minute injected I⁻ into the rat (Arriagada et al., 2015).
5. The U.S. EPA's Endocrine Disruptor Screening Program aims to use high-throughput assays and computational toxicology models to screen and prioritize chemicals that may disrupt the thyroid signaling pathway. Thyroid hormone biosynthesis requires active iodide uptake mediated by the sodium/iodide symporter (NIS). Monovalent anions, such as the environmental contaminant perchlorate, are competitive inhibitors of NIS, yet limited information exists for more structurally diverse chemicals. A novel cell line expressing human NIS, hNIS-HEK293TEPA, was used in a radioactive iodide uptake (RAIU) assay to identify inhibitors of NIS-mediated iodide uptake. The RAIU assay was optimized and performance evaluated with 12 reference chemicals comprising known NIS inhibitors and inactive

compounds. An additional 39 chemicals including environmental contaminants were evaluated, with 28 inhibiting RAIU over 20% of that observed for solvent controls. Cell viability assays were performed to assess any confounding effects of cytotoxicity. RAIU and cytotoxic responses were used to calculate selectivity scores to group chemicals based on their potential to affect NIS. RAIU IC₅₀ values were also determined for chemicals that displayed concentration-dependent inhibition of RAIU ($\geq 50\%$) without cytotoxicity. Strong assay performance and highly reproducible results support the utilization of this approach to screen large chemical libraries for inhibitors of NIS-mediated iodide uptake (Hallinger et al., 2017).

6. This study (Wang et al., 2018) applied a previously validated high-throughput approach to screen for NIS inhibitors in the ToxCast phase I library, representing 293 important environmental chemicals. Here 310 blinded samples were screened in a tiered-approach using an initial single-concentration (100 μ M) radioactive-iodide uptake (RAIU) assay, followed by 169 samples further evaluated in multi-concentration (0.001 μ M–100 μ M) testing in parallel RAIU and cell viability assays. A novel chemical ranking system that incorporates multi-concentration RAIU and cytotoxicity responses was also developed as a standardized method for chemical prioritization in current and future screenings. Representative chemical responses and thyroid effects of high-ranking chemicals are further discussed. This study significantly expands current knowledge of NIS inhibition potential in environmental chemicals and provides critical support to U.S. EPA's Endocrine Disruptor Screening Program (EDSP) initiative to expand coverage of thyroid molecular targets, as well as the development of thyroid adverse outcome pathways (AOPs).

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List of Key Events in the AOP

Event: 425: Decrease of Thyroidal iodide (<https://aopwiki.org/events/425>)

Short Name: Thyroidal Iodide, Decreased

Key Event Component

Process	Object	Action
iodide transport	iodide	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	KeyEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/176)	KeyEvent
Aop:188 - Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/188)	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

thyroid follicular cell

Organ term

Organ term

thyroid gland

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Pig	Pig	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
zebra fish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
Xenopus (Silurana) n. sp. tetraploid-1	Xenopus (Silurana) sp. new tetraploid 1	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=451443)

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	Moderate
Pregnancy	Moderate
During brain development	Moderate

Sex Applicability

Sex	Evidence
Mixed	Moderate

Various species express functional NIS encoded by the following genes: Human SLC5A5 (6528), Mouse Slc5a5 (114479), Rat Slc5a5 (114613), Zebrafish slc5a5 (561445), chicken SLC5A5 (431544), domestic cat SLC5A5 (101092587), dog SLC5A5 (484830), domestic guinea pig Slc5a5 (100714457), naked mole-rat Slc5a5 (101701995), cow SLC5A5 (505310), sheep SLC5A5 (101112315). The encoded protein is responsible for the uptake of iodine in tissues such as the thyroid and lactating breast tissue. The iodine taken up by the thyroid is incorporated into the metabolic regulators triiodothyronine (T3) and tetraiodothyronine (T4). Mutations in this gene are associated with thyroid dysmorphogenesis that significantly influences phenotypic expressions such as severity of hypothyroidism, goiter rates, and familial clustering demonstrating essentiality of NIS function to maintain TH status (Bakker et al., 2000; Spitzweg and Morris, 2010; Ramesh et al., 2016). Animal studies have also proven that iodine normalizes elevated adrenal corticosteroid hormone secretion and has the ability to reverse the effects of hypothyroidism in the ovaries, testicles and thymus in thyroidectomized rats (Nolan et al., 2000).

Key Event Description

Biological state: Iodine (I2) is a non-metallic chemical element which is required for the normal cellular metabolism. It is one of the essential components of the TH, comprising 65% and 58% of T4's and T3's weight, respectively and therefore it is crucial for the normal thyroid function. It is a trace element and a healthy human body contains 15-20 mg of iodine, most of which is concentrated in the thyroid gland (Dunn, 1998). Iodide (I-) that enters the thyroid gland remains in the free state only briefly and subsequently it binds to the tyrosine residues of thyroglobulin to form the precursors of the thyroid hormones mono-iodinated tyrosine (MIT) or di-iodinated tyrosine (DIT) (Berson and Yalow, 1955). The binding rate of iodide is 50-100% of the intra-thyroidal iodide pool, meaning that only a very small proportion of this element is free in the thyroid and this comes mainly by the deiodination of MIT and DIT.

The body is not able to produce or make iodine, thus the diet is the only source of this element. Iodine is found in nature in various forms, such as inorganic sodium and potassium salts (iodides and iodates), inorganic diatomic iodine and organic monoatomic iodine (Patrick, 2008). Thus, it is widely distributed in the environment but in many regions of the world the soil's iodine has been depleted due to different environmental phenomena. In these regions, the incidence of iodine deficiency is greatly increased (Ahad and Ganie, 2010).

The daily iodine intake of adult humans varies greatly due to the different dietary habits between the different regions on earth (Dunn, 1993). In any case, the ingested iodine is absorbed through the intestine and transported into the plasma to reach the thyroid gland. However, thyroid is not the only organ of the body that concentrates iodide. It has been shown that other tissues have also the ability of iodide concentration, such as the salivary glands, the gastric mucosa, the mammary glands and the choroid plexus, all of which express NIS, the iodine transporter protein (Jhiang et al., 1998; Cho et al., 2000).

Biological compartments: A sodium-iodide (Na/I) symporter pumps iodide (IO) actively into the cell, which previously has crossed the endothelium by largely unknown mechanisms. This iodide enters the follicular lumen from the cytoplasm by the transporter pendrin, in a purportedly passive manner. In the colloid, iodide (I-) is oxidized to iodine (IO) by an enzyme called thyroid peroxidase (TPO). IO is very reactive and iodinates the thyroglobulin at tyrosyl residues in its protein chain. In conjugation, adjacent tyrosyl residues are paired together. Thyroglobulin binds the megalin receptor for endocytosis back into the follicular cell. Proteolysis by various proteases liberates thyroxine (T4) and triiodothyronine molecules (T3), which enter the bloodstream where they are bound to thyroid hormone binding proteins, thyroxin binding globulin (TBG) which accounts for about 75% of the bound hormone. The adult thyroid absorbs 60-80 µg of iodide per day to maintain the thyroid homeostasis (Degroot, 1966). Inadequate amount of iodide results to deficient production of thyroid hormones, which consequently leads to an increase of TSH secretion and goiter, as compensating effect (Delange, 2000). On the other hand, excess iodide could also inhibit TH synthesis (Wolff and Chaikoff, 1948). The proposed mechanism for this latter effect is the possible formation of 2-iodohexadecanal that inhibits the generation of H2O2 and the subsequent oxidation of iodide in the thyroid follicular cells. The lack of oxidized free radicals of iodide affects the reaction with the tyrosine residues of Thyroglobulin (Tg) (Panneels et al., 1994). During pregnancy, the organism of the mother is also supporting the needs of the foetus and therefore the iodide requirements are greatly increased (Glinner, 1997). Additionally, small iodine concentrations have been found to have significant antioxidant effects that resembles to ascorbic acid (Smyth, 2003).

General role in biology: The most important role of iodine is the formation of the thyroid hormones (T4 and T3). The thyroid actively concentrates the circulating iodide through the basolateral membrane of the thyrocytes by the sodium/iodide symporter protein (NIS). The concentrated thyroid-iodine is oxidized in the follicular cells of the gland and consequently binds to tyrosines to form mono- or di-iodotyrosines (MIT and DIT respectively), being incorporated into thyroglobulin. This newly formed iodotyroglobulin forms one of the most important constituents of the colloid material, present in the follicle of the thyroid unit. If two di-iodotyrosine molecules couple together, the result is the formation of thyroxin (T4). If a di-iodotyrosine and a mono-iodotyrosine are coupled together, the result is the formation of tri-iodothyronine (T3). From the perspective of the formation of thyroid hormone, the major coupling reaction is the di-iodotyrosine coupling to produce T4.

AOP54

How it is Measured or Detected

The radioactive iodine uptake test, or RAIU test, is a type of scan used in the diagnosis of thyroid gland dysfunction (<http://www.thyca.org/pap-fol/rai/> (<http://www.thyca.org/pap-fol/rai/>); Kwee, et al., 2007). The patient swallows radioactive iodine in the form of capsule or fluid, and its absorption by the thyroid is studied after 4–6 hours and after 24 hours with the aid of a gamma scintillation counter. The percentage of RAIU 24 hours after the administration of radioiodide is the most useful, since this is the time when the thyroid gland has reached the plateau of isotope accumulation, and because it has been shown that at this time, the best separation between high, normal, and low uptake is obtained. The test does not measure hormone production and release but merely the avidity of the thyroid gland for iodide and its rate of clearance relative to the kidney.

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Event: 277: Thyroid hormone synthesis, Decreased (<https://aopwiki.org/events/277>)

Short Name: TH synthesis, Decreased

Key Event Component

Process	Object	Action
thyroid hormone generation	thyroid hormone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/42)	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/65)	KeyEvent
Aop:128 - Kidney dysfunction by decreased thyroid hormone (https://aopwiki.org/aops/128)	MolecularInitiatingEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	KeyEvent
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/159)	KeyEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/175)	KeyEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/176)	KeyEvent
Aop:188 - Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/188)	KeyEvent
Aop:192 - Pendrin inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/192)	KeyEvent
Aop:193 - Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/193)	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

thyroid follicular cell

Organ term

Organ term

thyroid gland

Evidence for Perturbation by Stressor**Overview for Molecular Initiating Event**

not applicable as this KE is not an MIE

Propylthiouracil

6-n-propylthiouracil is a common positive control

Methimazole

Methimazole is a very common positive control

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Pig	Pig	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)
Xenopus laevis	Xenopus laevis	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8355)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Decreased TH synthesis resulting from TPO or NIS inhibition is conserved across taxa, with *in vivo* evidence from humans, rats, amphibians, some fish species, and birds, and *in vitro* evidence from rat and porcine microsomes. Indeed, TPO and NIS mutations result in congenital hypothyroidism in humans (Bakker et al., 2000; Spitzweg and Morris, 2010), demonstrating the essentiality of TPO and NIS function toward maintaining euthyroid status. Though decreased serum T4 is used as a surrogate measure to indicate chemical-mediated decreases in TH synthesis, clinical and veterinary management of hyperthyroidism and Grave's disease using propylthiouracil and methimazole, known to decrease TH synthesis, indicates strong medical evidence for chemical inhibition of TPO (Zoeller and Crofton, 2005).

Key Event Description

The thyroid hormones (TH), triiodothyronine (T3) and thyroxine (T4) are thyrosine based hormones. Synthesis of TH is regulated by thyroid-stimulating hormone (TSH) binding to its receptor and thyroidal availability of iodine via the sodium iodide symporter (NIS). Other proteins contributing to TH production in the thyroid gland, including thyroperoxidase (TPO), dual oxidase enzymes (DUOX), and pendrin are also necessary for iodothyronine production (Zoeller et al., 2007).

The production of THs in the thyroid gland and resulting serum concentrations are controlled by a negatively regulated feedback mechanism. Decreased T4 and T3 serum concentrations activates the hypothalamus-pituitary-thyroid (HPT) axis which upregulates thyroid-stimulating hormone (TSH) that acts to increase production of additional THs (Zoeller and Tan, 2007). This regulatory system includes: 1) the hypothalamic secretion of the thyrotropin-releasing hormone (TRH); 2) the thyroid-stimulating hormone (TSH) secretion from the anterior pituitary; 3) hormonal transport by the plasma binding proteins; 4) cellular uptake mechanisms at the tissue level; 5) intracellular control of TH concentration by deiodinating mechanisms; 6) transcriptional function of the nuclear TH receptor; and 7) in the fetus, the transplacental passage of T4 and T3 (Zoeller et al., 2007).

TRH and the TSH primarily regulate the production of T4, often considered a "pro-hormone," and to a lesser extent of T3, the transcriptionally active TH. Most of the hormone released from the thyroid gland into circulation is in the form of T4, while peripheral deiodination of T4 is responsible for the majority of circulating T3. Outer ring deiodination of T4 to T3 is catalyzed by the deiodinases 1 and 2 (DIO1 and DIO2), with DIO1 expressed mainly in liver and kidney, and DIO2 expressed in several tissues including the brain (Bianco et al., 2006). Conversion of T4 to T3 takes place mainly in liver and kidney, but also in other target organs such as in the brain, the anterior pituitary, brown adipose tissue, thyroid and skeletal muscle (Gereben et al., 2008; Larsen, 2009).

Most evidence for the ontogeny of TH synthesis comes from measurements of serum hormone concentrations. And, importantly, the impact of xenobiotics on fetal hormones must include the influence of the maternal compartment since a majority of fetal THs are derived from maternal blood early in fetal life, with a transition during mid-late gestation to fetal production of THs that is still supplemented by maternal THs. In humans, THs can be found in the fetus as early as gestational weeks 10-12, and concentrations rise continuously until birth. At term, fetal T4 is similar to maternal levels, but T3 remains 2-3 fold lower than maternal levels. In rats, THs can be detected in the fetus as early as the second gestational week, but fetal synthesis does not start until gestational day 17 with birth at gestational day 22-23. Maternal THs continue to supplement fetal production until parturition. (see Howdeshell, 2002; Santisteban and Bernal, 2005 for review). *The ontogeny of TPO inhibition during development by environmental chemicals is a data gap.*

Decreased TH synthesis in the thyroid gland may result from several possible molecular-initiating events (MIEs) including: 1) Disruption of key catalytic enzymes or cofactors needed for TH synthesis, including TPO, NIS, or dietary iodine insufficiency. Theoretically, decreased synthesis of Tg could also affect TH production (Kessler et al., 2008; Yi et al., 1997). Mutations in genes that encode requisite proteins in the thyroid may also lead to impaired TH synthesis, including mutations in pendrin associated with Pendred Syndrome (Dossena et al., 2011), mutations in TPO and Tg (Huang and Jap 2015),

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and mutations in NIS (Spitzweg and Morris, 2010). 2) Decreased TH synthesis in cases of clinical hypothyroidism may be due to Hashimoto's thyroiditis or other forms of thyroiditis, or physical destruction of the thyroid gland as in radioablation or surgical treatment of thyroid lymphoma. 3) It is possible that TH synthesis may also be reduced subsequent to disruption of the negative feedback mechanism governing TH homeostasis, e.g. pituitary gland dysfunction may result in a decreased TSH signal with concomitant T3 and T4 decreases. 4) More rarely, hypothalamic dysfunction can result in decreased TH synthesis.

Increased fetal thyroid levels are also possible. Maternal Graves disease, which results in fetal thyrotoxicosis (hyperthyroidism and increased serum T4 levels), has been successfully treated by maternal administration of TPO inhibitors (c.f., Sato et al., 2014).

It should be noted that different species and different life stages store different amounts of TH precursor and iodine within the thyroid gland. Thus, decreased TH synthesis via transient iodine insufficiency or inhibition of TPO may not affect TH release from the thyroid gland until depletion of stored iodinated Tg. Adult humans may store sufficient Tg-DIT residues to serve for several months to a year of TH demand (Greer et al., 2002; Zoeller, 2004). Neonates and infants have a much more limited supply of less than a week.

How it is Measured or Detected

Decreased TH synthesis is often implied by measurement of TPO and NIS inhibition measured clinically and in laboratory models as these enzymes are essential for TH synthesis. Rarely is decreased TH synthesis measured directly, but rather the impact of chemicals on the quantity of T4 produced in the thyroid gland, or the amount of T4 present in serum is used as a marker of decreased T4 release from the thyroid gland (e.g., Romaldini et al., 1988). Methods used to assess TH synthesis include, incorporation of radiolabel tracer compounds, radioimmunoassay, ELISA, and analytical detection.

Recently, amphibian thyroid explant cultures have been used to demonstrate direct effects of chemicals on TH synthesis, as this model contains all necessary synthesis enzymes including TPO and NIS (Hornung et al., 2010). For this work THs was measured by HPLC/ICP-mass spectrometry. Decreased TH synthesis and release, using T4 release as the endpoint, has been shown for thiouracil antihyperthyroid drugs including MMI, PTU, and the NIS inhibitor perchlorate (Hornung et al., 2010).

TIQDT (Thyroxine-immunofluorescence quantitative disruption test) is a method that provides an immunofluorescent based estimate of thyroxine in the gland of zebrafish (Thienpont et al 2011). This method has been used for ~25 xenobiotics (e.g., amitrole, perchlorate, methimazole, PTU, DDT, PCBs). The method detected changes for all chemicals known to directly impact TH synthesis in the thyroid gland (e.g., NIS and TPO inhibitors), but not those that upregulate hepatic catabolism of T4.

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Event: 281: Thyroxine (T4) in serum, Decreased (<https://aopwiki.org/events/281>)

Short Name: T4 in serum, Decreased

Key Event Component

Process	Object	Action
regulation of hormone levels	thyroxine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/42)	KeyEvent
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/8)	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/65)	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity (https://aopwiki.org/aops/152)	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/159)	KeyEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/175)	KeyEvent

AOP54

AOP ID and Name	Event Type
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/176)	KeyEvent
Aop:194 - Hepatic nuclear receptor activation leading to altered amphibian metamorphosis (https://aopwiki.org/aops/194)	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
serum

Evidence for Perturbation by Stressor

Propylthiouracil

6-n-propylthiouracil is a classic positive control for inhibition of TPO

Perchlorate

Perchlorate ion (ClO⁻) is a classic positive control for inhibition of NIS

Methimazole

Classic positive control

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	<i>Mus musculus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
chicken	<i>Gallus gallus</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9031)
Xenopus laevis	<i>Xenopus laevis</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8355)
Pig	Pig	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

The overall evidence supporting taxonomic applicability is strong. THs are evolutionarily conserved molecules present in all vertebrate species (Hubert, 2000; Yen, 2001). Moreover, their crucial role in zebra fish (Thienpont et al., 2011), amphibian and lamprey metamorphoses is well established (Manzon and Youson, 1997; Yaoita and Brown, 1990; Furlow and Neff, 2006). Their existence and importance has also been described in many different animal and plant kingdoms (Eales, 1997; Heyland and Moroz, 2005), while their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of TH in the different species depends on the expression and function of specific proteins (e.g. receptors or enzymes) under TH control and may vary across species and tissues. As such extrapolation regarding TH action across species should be done with caution.

With few exceptions, vertebrate species have circulating T4 (and T3) that are bound to transport proteins in blood. Clear species differences exist in serum transport proteins (Dohler et al., 1979; Yamauchi and Isihara, 2009). There are three major transport proteins in mammals: thyroid binding globulin (TBG), transthyretin (TTR), and albumin. In adult humans, the percent bound to these proteins is about 75, 15 and 10 percent, respectively (Schusser 2000). In contrast, in adult rats the majority of THs are bound to TTR. Thyroid binding proteins are developmentally regulated in rats. TBG is expressed in rats until approximately postnatal day (PND) 60, with peak expression occurring during weaning (Savu et al., 1989). However, low levels of TBG persist into adult ages in rats and can be experimentally induced by hypothyroidism, malnutrition, or caloric restriction (Rouaze-Romet et al., 1992). While these species differences impact TH half-life (Capen, 1997) and possibly regulatory feedback mechanisms, there is little information on quantitative dose-response relationships of binding proteins and serum hormones during development across different species. Serum THs are still regarded as the most robust measurable key event causally linked to downstream adverse outcomes.

Key Event Description

All iodothyronines are derived from the modification of tyrosine molecules (Taurog, 2000). There are two biologically active thyroid hormones (THs) in serum, triiodothyronine (T3) and T4, and a few inactive iodothyronines (rT3, 3,5-T2). T4 is the predominant TH in circulation, comprising approximately 80% of the TH excreted from the thyroid gland and is the pool from which the majority of T3 in serum is generated (Zoeller et al., 2007). As such, serum T4 changes usually precede changes in other serum THs. Decreased thyroxine (T4) in serum results from one or more MIEs upstream and is considered a key biomarker of altered TH homeostasis (DeVito et al., 1999).

Serum T4 is used as a biomarker of TH status because the circulatory system serves as the major transport and delivery system for TH delivery to tissues. The majority of THs in the blood are bound to transport proteins (Bartalena and Robbins, 1993). In serum, it is the unbound, or 'free' form of the hormone that is thought to be available for transport into tissues. Free hormones are approximately 0.03 and 0.3 percent for T4 and T3, respectively. There are major species differences in the predominant binding proteins and their affinities for THs (see below). However, there is broad agreement that changes in serum concentrations of THs is diagnostic of thyroid disease or chemical-induced disruption of thyroid homeostasis (DeVito et al., 1999; Miller et al., 2009; Zoeller et al., 2007).

Normal serum T4 reference ranges can be species and lifestage specific. In rodents, serum THs are low in the fetal circulation, increasing as the fetal thyroid gland becomes functional on gestational day 17, just a few days prior to birth. After birth serum hormones increase steadily, peaking at two weeks, and falling slightly to adult levels by postnatal day 21 (Walker et al., 1980; Harris et al., 1978; Goldey et al., 1995; Lau et al., 2003). Similarly, in humans, adult reference ranges for THs do not reflect the normal ranges for children at different developmental stages, with TH concentrations highest in infants, still increased in childhood, prior to a decline to adult levels coincident with pubertal development (Corcoran et al. 1977; Kapelari et al., 2008). In some frog species, there is an analogous peak in thyroid hormones in tadpoles that starts around NF stage 56, peaks at Stage 62 and the declines to lower levels by Stage 56 (Sternberg et al., 2011; Leloup and Buscaglia, 1977).

How it is Measured or Detected

Serum T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone concentrations are clinically considered more direct indicators of T4 and T3 activities in the body, but in animal studies, total T3 and T4 are typically measured. Historically, the most widely used method in toxicology is radioimmunoassay (RIA). The method is routinely used in rodent endocrine and toxicity studies. The ELISA method is a commonly used as a human clinical test method. Analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates, though methods employing HPLC, liquid chromatography, immunoluminescence, and mass spectrometry are less common, but are becoming increasingly available (Hornung et al., 2015; DeVito et al., 1999; Baret and Fert, 1989; Spencer, 2013). It is important to note that thyroid hormones concentrations can be influenced by a number of intrinsic and extrinsic factors (e.g., circadian rhythms, stress, food intake, housing, noise) (see for example, Döhler et al., 1979).

Any of these measurements should be evaluated for the relationship to the actual endpoint of interest, repeatability, reproducibility, and lower limits of quantification using a fit-for-purpose approach (i.e., different regulatory needs will require different levels of confidence in the AOP). This is of particular significance when assessing the very low levels of TH present in fetal serum. Detection limits of the assay must be compatible with the levels in the biological sample. All three of the methods summarized above would be fit-for-purpose, depending on the number of samples to be evaluated and the associated costs of each method. Both RIA and ELISA measure THs by an indirect methodology, whereas analytical determination is the most direct measurement available. All these methods, particularly RIA, are repeatable and reproducible.

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Event: 280: Thyroxine (T4) in neuronal tissue, Decreased (<https://aopwiki.org/events/280>)

Short Name: T4 in neuronal tissue, Decreased

Key Event Component

Process	Object	Action
regulation of hormone levels	thyroxine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/42)	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/8)	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/65)	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity (https://aopwiki.org/aops/152)	KeyEvent

Stressors

Name
Methimazole
Propylthiouracil

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability			
Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
chicken	Gallus gallus	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9031)

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

THs are critical for normal brain development in most vertebrates, primarily documented empirically in mammalian species (Bernal, 2013). However, there is compelling data that demonstrates the need for TH in brain development for many other taxa, including: birds, fish and frogs (Van Herck et al., 2013; Denver, 1998; Power et al., 2001). The most well known non-mammalian action of TH is to induce metamorphosis in amphibians and some fish species. However, there is a fundamental difference in the mechanisms by which T3 affects amphibian metamorphosis vs its role in mammalian brain development (Galton, 1983). In the rat, brain development proceeds, even if defective, despite the absence of TH. By contrast, TH administration to tadpoles induces early metamorphosis, whereas in its absence, tadpoles grow to extremely large size, but the metamorphosis program is never activated (Galton, 1983).

Key Event Description

Thyroid hormones (TH) are present in brain tissue of most vertebrate species, and thyroxine (T4) is converted to triiodothyronine locally in this tissue. The amount of THs in brain is known to vary during development and to differ among brain regions (Calvo et al., 1990; Kester et al., 2004; Tu et al., 1999). In human cerebral cortex, T3 increases steadily from 13-weeks, reaching adult levels by 20 weeks post conception. This occurs despite very low and unchanging levels in fetal serum T3, when fetal serum T4 increases 3-fold over the same period. This indicates that T3 in fetal brain is locally generated from serum-derived T4 via the activity of deiodinases, primarily DIO2. DIO2 serves to convert T4 to T3. During this time in fetal development DIO3 activity, which converts T3 to the inactive reverse T3 (rT3), remains very low in cortex. In contrast, in other brain regions including hippocampus and cerebellum, T3 remains low throughout early and mid-gestation and corresponds with high activity of DIO3 in these brain regions. In late gestation and after birth, DIO3 levels drop in hippocampus and cerebellum with a corresponding increase in T3 concentrations (Kester et al., 2004).

A similar spatial and temporal profile of deiodinase activity and corresponding brain hormone concentrations has been observed in rodent brain (Calvo et al., 1990; Tu et al., 1999). In the rat, either whole brain or cortex have been preferentially assessed due to the low levels of hormones present and the small tissue volumes make quantification difficult. Brain T3 and T4 rise in parallel from gestational day 10 to gestational day 20 in rat. They are typically both quite low until gestational 17 with steep increases between GD18 and GD20 corresponding to the onset of fetal thyroid function (Calvo et al., 1990; Ruiz de Oña et al., 1988; Obergon et al., 1981). Just before birth, brain T3 and T4 concentrations are about one-third to one-half that of adult brain. Brain development in the early postnatal period in rat is roughly equivalent to the 3rd trimester in humans such that adult levels of T3 and T4 in brain are not reached in rodents until the 2nd-3rd postnatal week.

For THs to gain access to brain tissue they need to cross the blood brain barrier (BBB) which regulates the active transport of TH into neurons. Many transporter proteins have been identified, and the monocarboxylate transporters (Mct8, Mct10) and anion-transporting polypeptide (OATP1c1) show the highest degree of affinity towards TH and are prevalent in brain (Jansen et al., 2007; Mayer et al., 2014). Transporters express a distinct distribution pattern that varies by tissue and age (Friesema et al., 2005; Henneman et al., 2001; Visser et al., 2007; Heuer et al., 2005; Muller and Heuer, 2007). Although several transporters have been identified, current knowledge of cell specific profile of transporters is limited.

Most of the hormone transported across the blood brain barrier is in the form of T4, primarily though the cellular membrane transporters (e.g., OATP1c1 transporter) into the astrocyte (Visser and Visser, 2012; Sugiyama et al., 2003; Tohyama et al., 2004). Within the astrocyte, T4 is converted into T3 via the local activity of deiodinase 2 (DIO2) (Guadano-Ferraz et al., 1997). A small amount of T3 may cross the blood brain barrier directly via the T3-specific transporter, MCT8 (Heuer et al., 2005). Although in mature brain T3 derives partially from the circulation and from the deiodination of T4, in the fetal brain T3 is exclusively a product of T4 deiodination (Calvo et al., 1990; Grijota-Martinez et al., 2011). In both cases, only the required amount of T3 is utilized in neurons and the excess is degraded by the neuron-specific deiodinase DIO3 (Tu et al., 1999; St. Germain et al., 2009; Hernandez et al., 2010).

Both deiodinase and transporter expression in brain peak in different brain regions at different times in fetal and neonatal life (Kester et al., 2004; Bates et al., 1999; Muller and Heuer, 2014; Heuer, 2007). Collectively, these spatial and temporal patterns of transporter expression and deiodinase activity provide exquisite control of brain T3 available for nuclear receptor activation and regulated gene expression.

How it is Measured or Detected

Radioimmunoassays (RIAs) are commonly used to detect TH in the brain (e.g., Obregon et al., 1982; Calvo et al., 1990; Morse et al., 1996; Bansal et al., 2005; Gilbert et al., 2013). The method (and minor variants) is well established in the published literature. However, it is not available in a simple 'kit' and requires technical knowledge of RIAs, thus has not been used in most routine toxicology studies. Evaluations in neuronal tissue are complicated by the difficulty of the fatty matrix, heterogeneity of regions within the brain, and low tissue concentrations and small tissue amounts especially in immature brain. Most often whole brain homogenates are assessed, obfuscating the known temporal and regional differences in brain hormone present. Two analytical techniques, LC- and HPLC-inductively coupled plasma-mass spectrometry have recently been used to measure brain concentrations of TH. These techniques have proven capable of measuring very low levels in whole-body homogenates of frog tadpoles at different developmental stages (e.g., Simon et al., 2002; Tietge et al., 2010). The assay detects I-, MIT, DIT, T4, T3, and rT3. More recently, Wang and Stapleton (2010) and Donzelli et al. (2016) used liquid chromatography-tandem mass spectrometry for the simultaneous analysis of five THs including thyroxine (T4), 3,3',5-triiodothyronine (T3), 3,3',5-triiodothyronine (rT3; reverse T3), 3,3'-diiodothyronine (3,3'-T2), and 3,5-diiodothyronine (3,5-T2) in serum and a variety of tissues including brain. These analytical methods require expensive equipment and technical expertise and as such are not routinely used.

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Event: 381 : Reduced levels of BDNF (<https://aopwiki.org/events/381>)

Short Name: BDNF, Reduced

Key Event Component

Process	Object	Action
gene expression	brain-derived neurotrophic factor	decreased
secretion	brain-derived neurotrophic factor	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 (https://aopwiki.org/aops/13)	KeyEvent
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	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 (https://aopwiki.org/aops/12)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
neural cell

Domain of Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High
Sex Applicability	
Sex	Evidence
Mixed	High

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

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

Key Event Description

Biological state: BDNF belongs to a family of closely related neurotrophic factors named neurotrophins and is widely expressed in the developing and mature CNS. In the rodent cortex, postnatal BDNF expression is initially low but slowly increases to reach high levels around weaning. Therefore, BDNF expression peaks at a time when both structural and functional maturation of cortical circuitry occurs. During postnatal development, BDNF levels are dynamically regulated, in part by neuronal activity dependent mechanisms (Waterhouse and Xu, 2009). Glutamate has been shown to increase the transcription and release of BDNF. Indeed, BDNF is synthesized, stored and released from glutamatergic neurons (Lessmann et al., 2003).

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

In humans (Pruunsild et al., 2007), mBDNF is sequestered in platelets, consequently BDNF can reach all tissues and organs. Lymphocytic cells have been shown to express BDNF in vitro similarly to eosinophils, dendritic cells, and endothelial cells. The visceral and airway epithelium are also significant sources of BDNF. Female reproductive system including ovaries, placenta and uterus also express BDNF (Wessels et al., 2014).

General role in biology: The biological functions of mBDNF are mediated by binding to tyrosine kinase B (TrkB) receptor that leads to the activation of three major intracellular signalling pathways, including MAPK, PI3K and PLCγ1 (Soulé et al., 2006). TrkB-mediated signaling regulates gene transcription in the nucleus through the activation of several transcription factors. These genes are involved in neurite outgrowth, synaptogenesis, synapse maturation and stabilization (Pang et al., 2004; Lu et al., 2005; Nelson and Alkon, 2014).

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

How it is Measured or Detected

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

No OECD methods are available to measure BDNF protein and mRNA levels. Measuring BDNF levels changes in the brain, especially when low, at the boarder to be significant are technically difficult. Depending on the tissue or fluid measurements distinct methods are used.

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

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

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

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

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Event: 851: Decrease of GABAergic interneurons (<https://aopwiki.org/events/851>)

Short Name: GABAergic interneurons, Decreased

Key Event Component

Process	Object	Action
abnormal neuron morphology		abnormal

AOPs Including This Key Event

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

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
GABAergic interneuron

Domain of Applicability

Taxonomic Applicability			
Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
<i>Caenorhabditis elegans</i>	<i>Caenorhabditis elegans</i>	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=6239)

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

GABAergic interneurons play a vital role in the wiring and circuitry of the developing nervous system of all organisms, both invertebrates and vertebrates (Hensch, 2005; Owens and Kriegstein, 2002; Wang et al., 2004). However, restricted expression of GABA in a considerable population of neurons is observed in the non-vertebrate animals. A nematode *Caenorhabditis elegans* has 302 neurons, among them, 26 cells are GABAergic (Sternberg and Horvitz, 1984; McIntire et al., 1993). Another nematode *Ascaris* has 26 GABAergic neurons (Obata, 2013). GAD, VGAT, GABA receptors and GABA-system-specific molecules are analogous to those of vertebrates. Except for one interneuron, GABAergic neurons are connected with muscle cells and exert direct inhibitory, sometimes excitatory, control on locomotion, defecation and foraging. The muscle innervation of both excitatory and inhibitory axons is maintained also in Crustacea (Obata, 2013).

Key Event Description

Biological state

The GABA-mediated depolarizing effects at the post-synaptic neurons in early development are well documented (Ben-Ari, 2014) and have been greatly correlated with the emergence of spontaneous network activity, which is the first neuronal activity of the brain (Voigt et al., 2001; Opitz et al., 2002). This spontaneous network activity is characterized by synchronous bursts of action potentials and concomitant intracellular calcium transients in large group of cells and it has been proposed to have functional role during the synaptogenesis and the formation of connections within the neuronal network (Wang and Kriegstein, 2010; Ben Ari et al., 2007; Blankenship and Feller, 2010).

One of the milestones at the critical stage of brain development is the switch of the GABAergic signalling from depolarizing early in life to a more conventional hyperpolarizing inhibition on maturation (Ben-Ari et al., 2007). This developmental GABAergic switch is mainly driven by the expression change of the predominant potassium-chloride co-transporters (KCC2 and NKCC1) around this period that results in a shift from high to low intracellular Cl⁻ concentration at the post-synaptic neurons (Lu et al., 1999).

Biological compartments

GABAergic interneurons are a heterogeneous group of neuronal cells that consist only 10 to 20% of the total neuronal population (Aika et al., 1994; Halasy and Somogyi, 1993). They are characterized by aspiny dendrites and the release of GABA neurotransmitter, which makes them the main inhibitory source in the adult central nervous system (CNS) (Markram et al., 2004). A hallmark of interneurons is their structural and functional diversity. Many different subtypes have been identified in the cortex and hippocampus, but a global classification in specific categories is difficult to be established due to the variable morphological and functional properties (Klausberger and Somogyi, 2008; DeFelipe et al., 2013). The interneurons can be primarily identified by their characteristic morphology, which would divide them into 4 basic groups: basket cells, chandelier cells, bouquet cells and bitufted cells. However, a broader classification of these cells would require at least the following criteria: 1) morphology of soma, axonal and dendritic arbors; 2) molecular markers including but not restricted to calcium binding proteins (parvalbumin, calbindin, calretinin) and neuropeptides (e.g., Vasoactive Intestinal Peptide [VIP], reelin, somatostatin); 3) postsynaptic target cells; and 4) functional characteristics (Ascoli et al., 2008). They are neither motor nor sensory neurons, and also differ from projection neurons which send their signals to more distant locations.

GABAergic interneurons are broadly present throughout the CNS, although telencephalic structures, such as the cerebral cortex and hippocampus, show the most abundant quantities of this neurotransmitter (Jones 1987). Complex interconnections between GABAergic interneurons and pyramidal cells shape the responses of pyramidal cells to incoming inputs, prevent runaway excitation, refine cortical receptive fields, and are involved in the timing and synchronisation of network oscillations (Wehr and Zador, 2003; Markram et al., 2004; LeMaqueresse and Monyer, 2013; Hu et al., 2014).

General role in biology

Inhibitory GABAergic interneurons of the adult nervous system play a vital role in neural circuitry and activity by regulating the firing rate of target neurons (reducing neuronal excitability). In vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors of both pre- and postsynaptic neuronal processes. Released neurotransmitter typically acts through postsynaptic GABA_A ionotropic receptors in order to trigger a neuronal signalling pathway. This binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell (https://en.wikipedia.org/wiki/Cell_biology) or positively charged potassium ions out of the cell. This action results in a negative change in the transmembrane potential (https://en.wikipedia.org/wiki/Transmembrane_potential), usually causing hyperpolarization (https://en.wikipedia.org/wiki/Hyperpolarization_biology).

During early brain development GABA mediates depolarization that has recently been shown to promote excitatory synapse formation by facilitating NMDA receptor activation in cortical pyramidal neurons (Wang and Kriegstein, 2008). GABAergic signalling has the unique property of "ionic plasticity", which is dependent on short-term and long-term concentration changes of Cl⁻ and HCO₃⁻ in the postsynaptic neurons. The intracellular ion concentrations are largely modified in the course of brain development corresponding to the operation and functional modulation of ion transporters, such as the K-Cl co-transporter 2 (KCC2) and the Na-K-Cl co-transporter 1 (NKCC1) (Blaesse et al., 2009; Blankenship and Feller, 2010).

GABA plays an important role as the first excitatory transmitter during embryogenesis and it has been shown to affect neurogenesis, differentiation, migration, and integration of developing neurons into neuronal circuits (LoTurco et al., 1995; Heck, et al., 2007).

The effects of GABA being depolarizing are also important in the adult brain, as it has impact on synaptic plasticity and is strongly correlated with seizures (Baram and Hatalski, 1998; Ben-Ari et al., 2012). If GABAergic interneuron function breaks down, excitation takes over, leading to seizures and failure of higher brain functions (Westbrook, 2013).

How it is Measured or Detected

Parvalbumin (PV) is a marker of GABAergic interneurons (https://en.wikipedia.org/wiki/Interneuron) that can be identified by immunohistochemistry. GABA or GAD can be used for identification and morphometric analysis of the GABAergic neuronal population (Voigt et al., 2001; De Lima et al., 2007), with the use of anti-GABA antibodies. Protein levels on interneurons can be measured by commercial available antibody sandwich ELISA kits, Western blotting, immunohistochemistry and immunofluorescence and mRNA levels is possible to be measured with RT-PCR, with the use of the primers in interest each time.

Calcium imaging experiments is the most common way to detect the depolarizing action of neurons, as this is correlated with a transient increase in intracellular calcium (Voigt et al., 2001). The local application of GABA agonist, muscimol, during the calcium imaging has been used the last decades in order to investigate the developmental effects of GABA in the post-synaptic neurons (Owens et al., 1996; Ganguly et al., 2001; Baltz et al., 2010; Westerholz et al., 2013).

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Event: 385: Decrease of synaptogenesis (<https://aopwiki.org/events/385>)

Short Name: Synaptogenesis, Decreased

Key Event Component

Process	Object	Action
synapse assembly	synapse	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 (https://aopwiki.org/aops/13)	KeyEvent
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
neuron

Domain of Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

The mechanisms governing synapse formation is considered conserved among both vertebrates and invertebrates (Munno and Syed, 2003). Invertebrates have served as simple animal models to study synapse formation. Indeed, Colón-Ramos (2009) has recently reviewed the early developmental events that take place in the process of synaptogenesis pointing out the importance of this process in neural network formation and function. The experimental evaluation of synaptogenesis has been performed using invertebrates and in particular *C. elegans* and *Drosophila* as well as vertebrates (Colón-Ramos, 2009).

This vulnerable period of synaptogenesis appears to happen in different developmental stages across species. For example, in rodents primarily synaptogenesis occurs during the first two weeks after birth (Bai et al., 2013). For rhesus monkeys, this period ranges from approximately 115-day gestation up to PND 60 (Bai et al., 2013). In humans, it starts from the third trimester of pregnancy and continues 2-3 years following birth (Bai et al., 2013).

Key Event Description

Biological state: Synaptogenesis is a multi-step process that is crucial for brain development and involves the formation of synapses. It follows axonal migration, at which stage presynaptic and postsynaptic differentiation occurs (Garner et al., 2002). "Synaptic assembly" that refers to the gathering of the appropriate components and "synaptic formation" that is defined by the mechanisms involved in recruitment of molecules required for differentiation, stabilization and maturation of synapse, are the main phases that characterise synaptogenesis (Colón-Ramos, 2009). Elimination is a physiological step involved in synaptogenesis regarding the synapses that fail to get stabilised and mature.

The first step is the recognition and the establishment of contact between an axon and a dendritic spine in which pre- and postsynaptic neurons play important role. The presynaptic differentiation occurs followed by excretion of neurotransmitters that bind to appropriate receptors located on the target spine. However, a postsynaptic neuron does not passively receive guidance from a presynaptic axon but are the same dendritic filopodia that gradually are transformed into spines that select and engage their presynaptic neurons. The transformation of dendritic filopodia into dendritic spines that involves the expression of the whole postsynaptic machinery such as postsynaptic density (PSD), receptor subunits, scaffolding proteins and actin cytoskeleton, is the first step to give nascent synapses. However, to become functional and mature these synapses need an important number of cell-cell interactions, including stimulation from glutamatergic synapses as well as the influence of neurotrophic factors (Munno and Syed, 2003).

However, all this is true for glutamatergic synapses because GABAergic synapses do not appear in dendritic spines, but rather form on dendritic shafts, nerve cell somata and axon initial segments. These inhibitory synapses besides their distinct location are also structurally different compared to excitatory synapses (reviewed in Gatto and Broadie, 2010).

Biological compartments: Synaptogenesis is spatially and temporally strictly controlled process. It does not happen in a uniform way in all brain regions and there are important differences between the times of appearance of the main two types of synapses (reviewed in Erecinska et al., 2004). For example, in rat hippocampus excitatory synapses are well established or fully mature within the two first postnatal weeks, whereas inhibitory synapses cannot be found prior to PND 18, after which it increases steadily to reach adult levels at PND 28. In addition, in rat neostriatal neurons the excitatory responses to both cortical and thalamic stimuli can be observed by PND 6, but the long-lasting hyperpolarization and late depolarization is never seen before PND 12.

Structural remodelling of synapses and formation of new synaptic contacts has been postulated as a possible mechanism underlying the late phase of long-term potentiation (LTP), a form of plasticity which is involved in learning and memory. LTP induction results in a sequence of morphological changes consisting of a transient remodelling of the postsynaptic membrane followed by a marked increase in the proportion of axon terminals contacting two or more dendritic spines. Three-dimensional reconstruction revealed that these spines arose from the same dendrite. As pharmacological blockade of LTP prevented these morphological changes, it is suggested that LTP is associated with the formation of new, mature and probably functional synapses contacting the same presynaptic terminal and thereby duplicating activated synapses (Erik et al., 2006).

In human, synaptogenesis does not happen at the same time in all brain regions, as the prefrontal cortex lags behind in terms of synapse formation compared to the auditory and visual cortices. In contrast, synaptogenesis appears to proceed concurrently in different brain areas for rhesus monkey (Erecinska et al., 2004).

General role in biology: The period of rapid synaptogenesis or the so-called brain growth spurt is considered one of the most important processes that take place during brain development (Garner et al., 2002). This process is crucial not only in neurodevelopment but also plays a vital role in synaptic plasticity, learning and memory and adaptation throughout life. Without this process no complex brain network can be established as synapse is the fundamental unit of connectivity and communication between neurons (Tau and Peterson, 2010). Cell adhesion represents the most direct way of coordinating synaptic connectivity in the brain. Recent evidence highlights the importance of a trans-synaptic interaction between postsynaptic neuroligins and presynaptic neurexins. These transmembrane molecules bind each other extracellularly to promote adhesion between dendrites and axons, facilitating synapse establishment (Dean and Dresbach, 2006). Furthermore, the number of excitatory versus inhibitory synapses created at single neuron dictates neuronal excitability and function (Schummers et al., 2002).

How it is Measured or Detected

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

There is no OECD advised method for measuring synaptogenesis.

Anatomical methods can be used to structurally estimate the number of excitatory or inhibitory synapses. Immunostaining can be employed with specific antibodies that recognize vesicular glutamate transporters (VGATs) and the postsynaptic density protein-95 kDa (PSD-95) that are characteristic of excitatory synapses, while inhibitory synapses are identified by the presence of the vesicular GABA (VGAT) and vesicular inhibitory amino acid (VIAAT) transporters and the postsynaptic adaptor protein gephyrin (Gatto and Broadie, 2010). There are commercial available synaptogenesis assay kits that rely on the immunostaining of cells with MAP-2, PSD-95 and synaptophysin. Some other presynaptic (Bassoon) and postsynaptic (ProSAP1/Shank2) markers have been suggested and showed to correlate well with the ultrastructural studies in cultured hippocampus primary cells (Grabrucker et al., 2009). Electron microscopy can also be applied to assess the prevalence of excitatory and inhibitory synapses amongst convergent contacts (Megias et al., 2001). Recently, a high content image analysis based on RNAi screening protocols has been suggested as a useful tool to create imaging algorithm for use in both *in vitro* and *in vivo* synaptic punctae analysis (Nieland et al., 2014).

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Event: 386: Decrease of neuronal network function (<https://aopwiki.org/events/386>)

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 (https://aopwiki.org/aops/13)	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1 (https://aopwiki.org/aops/78)	KeyEvent
Aop:90 - Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2 (https://aopwiki.org/aops/90)	KeyEvent
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	KeyEvent
Aop:17 - 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)	KeyEvent

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
brain

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mice	<i>Mus sp.</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)
cat	<i>Felis catus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9685)

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 -aminobutyric acid A (GABA A)-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 glutamatergic 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 AMPA R -mediated postsynaptic currents recorded in hippocampal CA1. Consistent, the deletion of NR3A accelerates the expression of the glutamate receptor subunits NR1, NR2A, and GluR1 suggesting 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).

AOP54

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List of Adverse Outcomes in this AOP

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

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 (https://aopwiki.org/aops/13)	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. (https://aopwiki.org/aops/48)	AdverseOutcome
Aop:54 - Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	AdverseOutcome
Aop:77 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1 (https://aopwiki.org/aops/77)	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1 (https://aopwiki.org/aops/78)	KeyEvent
Aop:87 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure (https://aopwiki.org/aops/87)	KeyEvent
Aop:88 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure via abnormal role change within caste (https://aopwiki.org/aops/88)	KeyEvent
Aop:89 - Nicotinic acetylcholine receptor activation followed by desensitization contributes to abnormal foraging and directly leads to colony loss/failure (https://aopwiki.org/aops/89)	KeyEvent
Aop:90 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony loss/failure 2 (https://aopwiki.org/aops/90)	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 (https://aopwiki.org/aops/12)	AdverseOutcome
Aop:99 - Histamine (H ₂) receptor antagonism leading to reduced survival (https://aopwiki.org/aops/99)	KeyEvent
Aop:17 - 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)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

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

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

Life Stage Applicability

Life Stage	Evidence
During brain development	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, visuo-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 Guijarro, 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 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 Osterrieth Complex Figure (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 neuropsychological 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: Text from LaLone et al. (2017) Weight of evidence evaluation of a network of adverse outcome pathways linking activation of the nicotinic acetylcholine receptor in honey bees to colony death. *Science of the Total Environment* 584-585, 751-775: "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). 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 and Sandoz, 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 426) 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: 442: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Thyroidal Iodide, Decreased (<https://aopwiki.org/relationships/442>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	adjacent	High	High
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	<i>Mus musculus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Xenopus laevis laevis	<i>Xenopus laevis laevis</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=443947)

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

Empirical evidence comes from in vitro works using rat follicular cells (Cianchetta et al., 2010; Waltz et al., 2010; Lecat-Guillem et al., 2007; 2008; Lecat-Guillem et al., 2008b), human in vitro cell models (Wen et al., 2016) and in vivo data (Amiagada et al. 2015), as well as Xenopus oocytes (Lindenthal et al., 2009) and Zebrafish (Thienpont et al., 2011).

Key Event Relationship Description

NIS is a membrane protein implicated in iodide uptake into the follicular cells of the thyroid. Other large anions can be also bound by NIS and inhibit accumulation of iodide into the thyroid by competing binding with iodide (Wolff, 1964).

Evidence Supporting this KER

Biological Plausibility

NIS is a membrane bound glycoprotein and its main physiological function is to transport one iodide ion along with two sodium ions across the basolateral membrane of thyroid follicular cells. It uses the sodium gradient generated by the Na⁺/K⁺ ATPase for the active transport of iodide into the thyrocytes (Eskandari et al., 1997). Extensive studies on NIS protein have identified 14 different mutations and each one of them is related to Iodide Transport Deficiencies (ITD) (reviewed in Spitzweg and Morris, 2010). Most of these mutations have been characterized and it is well known that they even lead to the synthesis of truncated protein (Pohlenz et al., 1997; Pohlenz et al., 1998), partial deletions (Kosugi et al., 2002; Tonacchera et al., 2003; Montanelli et al., 2009) or substitutions of amino acids (Matsuda and Kosugi, 1997; Kosugi et al., 1999; Szinnai et al., 2006) that eventually result in total or partial NIS dysfunction. While most of the NIS mutants have been further investigated and the functional relationship between the NIS dysfunction and ITD is well established (reviewed in Darrouzet et al., 2014; Portulano et al., 2014), the exact structural relationship between mutated NIS and ITD still needs to be elucidated and the molecular modelling of the protein would greatly benefit these studies. At the same time, causative link between iodide deficiency, thyroid hormones, and neurodevelopment defects is well documented (Gilbert et al., 2009).

Recent revision of the affinity constant for perchlorate binding to the NIS symporter based on in vitro and human in vivo data, performed by refitting published in vitro data, in which perchlorate-induced inhibition of iodide uptake via the NIS was measured, yielding a Michaelis-Menten kinetic constant (K_m) of 1.5 μ M, showed that a 60% lower value for the K_m, equal to 0.59 μ M. Substituting this value into the PBPK model for an average adult human significantly improved model agreement with the human RAIU data for exposures <100 μ g kg⁻¹ day⁻¹ (Schlosser PM, 2016).

The effects of maternal hypothyroidism could also contribute to this KER. During pregnancy TH requirements increase, particularly during the first trimester (Alexander et al. 2004; Leung et al. 2010), due to higher concentrations of thyroxine-binding globulin, placental T4 inner-ring deiodination leading to the inactive reverse T3 (rT3), and transfer of small amounts of T4 to the foetus (during the first trimester foetal thyroid function is absent). Moreover, glomerular filtration rate and clearance of proteins and other molecules are both increased during pregnancy, possibly causing increased renal iodide clearance and a decreased of circulating plasma iodine (Gilioer, 1997). Thus, even though the foetal thyroid can trap iodide by about 12 week of gestation (Fisher and Klein, 1981), high concentrations of maternal perchlorate may potentially decrease thyroidal iodine available to the foetus by inhibiting placental NIS (Leung et al. 2010).

Consequences of TH deficiency depend on the developmental timing of the deficiency (Zoeller and Rovet, 2004). For instance, if the TH deficiency occurs during early pregnancy, offspring show visual attention, visual processing and gross motor skills deficits, while if it occurs later, offspring may show subnormal visual and visuospatial skills, along with slower response speeds and motor deficits. If TH insufficiency occurs after birth, language and memory skills are most predominantly affected (Zoeller and Rovet, 2004).

There are limited data regarding low-level environmental perchlorate exposure and maternal thyroid function during pregnancy. A Chilean study found no increases in TSH or decreases in free thyroxine or urinary iodine concentrations in pregnant women living in three areas (all of which had more than adequate mean urinary iodine levels) with long-term environmental perchlorate exposure (Téllez Téllez et al. 2005). A follow-up analysis of this cohort also confirmed the lack of association between individual urinary iodide or perchlorate concentrations and thyroid function in the pregnant women (Gibbs and Van Landingham, 2008). Studies of large cohorts of first-trimester pregnant women from the U.S., Europe and Argentina found that environmental perchlorate exposure did not affect maternal thyroid function (Pearce et al. 2009).

Empirical Evidence

Many studies have shown inhibition of radioactive iodide uptake by using different cell models and assays. However, there have been identified only few specific NIS inhibitors up to date, while all the others are thought to act through different inhibitory mechanisms. Monovalent anions, others than iodide, are also transported by NIS but Nitrate (NO₃⁻), thiocyanate (SCN⁻), perchlorate (ClO₄⁻), dysidenin and aryltrifluoroborates are of particular dietary and environmental importance (Jones et al., 1996; Tonacchera et al., 2004; De Groef et al., 2006).

Recent revision of the affinity constant for perchlorate binding to the NIS symporter based on in vitro and human in vivo data, performed by refitting published in vitro data, in which perchlorate-induced inhibition of iodide uptake via the NIS was measured, yielding a Michaelis-Menten kinetic constant (K_m) of 1.5 μ M, showed that a 60% lower value for the K_m, equal to 0.59 μ M. Substituting this value into the PBPK model for an average adult human significantly improved model agreement with the human RAIU data for exposures <100 μ g kg⁻¹ day⁻¹ (Schlosser PM, 2016).

There are many studies showing the effect of inhibition of NIS on thyroidal iodide uptake:

- **Cianchetta et al., 2010** For this study the rat FRTL5 thyroid cell line endogenously expressing NIS, and the monkey kidney fibroblast-like cells (COS-7) transfected with hNIS were used. NIS functionality was assessed with the use of the Yellow Fluorescent Protein (YFP) variant YFP-H148Q/I152L, a genetically encodable biosensor of intracellular perchlorate concentration monitored by real-time fluorescence microscopy. Decrease of YFP-H148Q/I152L fluorescence in FRTL5 cells occurs as a result of NIS-mediated uptake and binding to the intracellular fluorochrome (Rhoden et al., 2007). The biosensor was used to compare the kinetics of iodide and perchlorate transport by NIS, and to assess the ability of perchlorate to inhibit iodide transport. Additionally, perchlorate was shown to inhibit NIS function (competitive inhibition) by preventing iodide-induced changes in fluorescence of FRTL5 cells. Perchlorate caused a concentration-dependent inhibition of iodide uptake in the initial influx rate (IC₅₀=1.6 μ M) and in the intracellular concentration of iodide (IC₅₀=1.1 μ M). Also, both perchlorate and iodide (1–1000 μ M) induced concentration-dependent decreases in YFP-H148Q/I152L fluorescence in COS-7 cells expressing hNIS, but had no effect (< 2%) in COS-7 cells lacking hNIS. Additionally, perchlorate induced a significantly smaller decrease in fluorescence (10.6% at 1 mM) than iodide (31.8% at 1 mM iodide). Thus, it was confirmed that the reduction of fluorescence was due to NIS-mediated anion transport into the cells, excluding non-specific effects.

- **Tonacchera et al., 2004** Chinese hamster ovary (CHO) cell line had been stably transfected with human NIS and the measurement of iodide uptake was performed with the use of radioactive iodide uptake (RAIU) method. It was shown that the inhibition of iodide uptake was dose-dependent when using the known NIS inhibitors (ClO₄⁻, NO₃⁻, SCN⁻). Additionally, unlabeled I⁻ (non-¹²⁵I) was used to investigate the inhibition level of radioiodide uptake and to compare it with the potency of the other monoions, which are known NIS inhibitors. The IC₅₀ values for the studied monoions were the following: ClO₄⁻: IC₅₀ was 1.22 μ M; SCN⁻: IC₅₀ was 18.7 μ M; NO₃⁻: IC₅₀ was 293 μ M; I⁻: IC₅₀ was 36.6 μ M. Finally, the present study investigated the joint effects of simultaneous exposure to multiple RAIU inhibitors, by generating multiple dose-response curves in the presence of fixed concentrations of inhibitors. The results of those experiments indicated a competition between the four anions with similar size for access to the binding sites of the NIS. The prediction model developed in this study, actually suggests that thyroidal iodide uptake is approximately proportional to iodide nutrition for any fixed inhibitor concentration, answering to the question whether dietary iodide can modulate the inhibitory effects of the known environmental goitrogens.

- Waltz et al., 2010 Measurement of iodide uptake was performed with a non-radioactive method. By using the rat thyroid low –serum 5 (FRTL5) cells, which endogenously express NIS, a spectrophotometric assay was developed and the iodide accumulation was determined based on the catalytic reduction of yellow cerium to colorless cerium in the presence of arsenious acid (Sandell-Kolthoff reaction). A dose-dependent inhibition of iodide uptake was shown. The IC₅₀ values for the studied compounds were the following: Sodium perchlorate (NaClO₄): IC₅₀ was 0.1 μ M Sodium thiocyanate (NaSCN): IC₅₀ was 12 μ M Sodium nitrate (NaNO₃): IC₅₀ was 800 μ M Sodium Tetrafluoroborate (NaBF₄): IC₅₀ was 1.2 μ M

- Lecat-Guillet et al., 2007; 2008a A fully automated radioiodide uptake assay was developed and some known NIS inhibitors were tested. A dose-dependent inhibition of iodide uptake was shown. The IC₅₀ values for the studied compounds were the following: Sodium perchlorate (NaClO₄): IC₅₀ was 1 μ M; Sodium thiocyanate (NaSCN): IC₅₀ was 250 μ M; Sodium Tetrafluoroborate (NaBF₄): IC₅₀ was 0.75 μ M. Additionally, a library of 17020 compounds was screened for the identification of new human NIS inhibitors. The identification was based on the magnitude of changes in iodide uptake using Human Embryonic Kidney 293 (HEK293) cells, stably transfected with the hNIS. The same experiments and with similar results were also performed in rat thyroid derived cells (FRTL5), which endogenously express NIS. Compounds that inhibited iodide uptake in a time-dependent manner were considered to act through direct NIS inhibition. In contrast, those compounds that had a delayed effect on iodide uptake were thought to act through a sodium gradient disruption system resulting in indirect inhibition of iodide transport. Perchlorate was used as a positive control in these experiments and, as expected, it blocked iodide uptake immediately and totally throughout the experiment. Dysidenin was also used as a control and the IC₅₀ value identified was 2 μ M. All the compounds that were used for these experiments were small drug-like molecules that have not been detected in the environment and they were named as ITBs (Iodide Transport Blockers).

- Lecat-Guillet et al., 2008b With the same fully automated radioiodide uptake assay, as described above, new NIS inhibitors were also identified. The organotrifluoroborate (BF3–) was found to inhibit iodide uptake with an IC₅₀ value of 0.4 μ M using rat-derived thyroid cells (FRTL5). The biological activity is rationalized by the presence of the ion BF3– as a minimal binding motif for substrate recognition at the iodide binding site.

- Lindenthal et al., 2009 With the use of a patch-clamp technique an analysis of the NIS inhibitors identified by Lecat-Guillet et al., 2008 (named ITB-1 to ITB-10 for "Iodide Transport Blockers") was evaluated in Xenopus oocytes expressing NIS to further assess the inhibitory effect of those molecules specifically on NIS activity. Four of those molecules (ITB-3, ITB-9, ITB-5 and ITB-4) were identified as the most potent, non-competitive NIS inhibitors. The effects of dysidenin were also analyzed with the same technique, as it had been reported to be a specific inhibitor of NIS (Vroye et al., 1998). It was found that dysidenin (50 μ M) induced a rapid and reversible inhibition of the iodide (about 40%) of induced current in mNIS-expressing oocytes, but did not evoke any currents in the absence of iodide, suggesting that this effect was due to the inhibition of NIS activity.

- Greer et al., 2002 In human studies, potassium perchlorate was used to predict inhibition of thyroidal iodide uptake by applying the RAIU method. Greer et al., tested body weight adjusted doses of potassium perchlorate and an assessment of RAIU uptake was performed on day 2 and day 14 of treatment and 24 h following treatment termination (on day 15). The NOEL value for inhibition of thyroidal uptake was 0.007 mg/kg-day, while the true NEL value was estimated to be 0.0052 and 0.0064 mg/kg-day. According to the dose-response inhibition of iodide uptake the maximum percentage of iodide inhibition at the doses of 0.0052 and 0.0064 mg/kg-day is 8.3-9.5%, which is physiologically insignificant for a person with dietary sufficient iodine intake.

- Wen et al., 2016 By using human MCF-7 cells, a breast adenocarcinoma cell line, which express inducible NIS in the presence of all-trans retinoic acid (ATRA) it has been shown that inhibition of sterol regulatory element-binding proteins (SREBP) maturation by treatment with 25-hydroxycholesterol (5 μ M) for 48 hr reduced ATRA (1 μ M)-induced mRNA concentration of NIS and decreased iodide uptake by approximately 20%. This study showed for the first time that the NIS gene and iodide uptake are regulated by SREBP in cultured human mammary epithelial cells.

- Arriagada et al. 2015 This study showed that 2 hr or 5 hr exposure to excess I⁻ (100 μ M) respectively in FRTL-5 cells and in ex-vivo rat thyroid gland (removed after single in vivo i.p. injection of 100 μ M of I⁻ in 500 μ L of distilled water, and analysis of ¹²⁵I thyroid uptake), induced inhibition of I⁻ uptake through the NIS (~ 30% uptake inhibition after 5 hr in vivo), a process known as the Wolff-Chaikoff effect, which was not associated with a decrease of NIS expression or a change in NIS localization. Incubation of FRTL-5 cells with excess I⁻ for 2 hr increased hydrogen peroxide generation. Also incubation with hydrogen peroxide (100 μ M) decreased NIS-mediated I⁻ transport, effect that was reverted by ROS scavengers.

Uncertainties and Inconsistencies

The thyroid system is quite complex and therefore some inconsistent results have been produced by recent studies. For example, it has been observed in healthy volunteers that a 6-month exposure to perchlorate at doses up to 3 mg/d (low doses) had no effect on thyroid function, including inhibition of thyroid iodide uptake as well as serum levels of thyroid hormones, TSH, and Tg (Braverman et al., 2006). However, this study was limited by the small sample size and is obviously underpowered.

The review by Charnley (2008) examines a number of studies where association between perchlorate environmental (low) exposure and thyroid effects were analysed and many inconsistent conclusions have been drawn. For instance, no correlations were found between TH serum levels and urinary iodine concentrations among women exposed to perchlorate participating in the 2000-2001 National Health and Nutrition Examination Survey (NHANES). Available evidence does not support a causal relationship between changes in TH levels and current environmental levels of perchlorate exposure, but does support the conclusion that the US Environmental Protection Agency's reference dose (RfD) for perchlorate is conservatively health-protective. However, potential perchlorate risks are unlikely to be distinguishable from the ubiquitous background of naturally occurring substances present at much higher exposures that can affect the thyroid via the same biological mode of action as perchlorate, such as nitrate and thiocyanate. Therefore, risk management approaches that account for both aggregate and cumulative exposures and that consider the larger public health context in which exposures are occurring are desirable.

Additionally, a more comprehensive study by Pearce et al. (2010) conducted during 2002-2006 on 22,000 women at less than 16-week gestation showed that while low-level perchlorate exposure was ubiquitous in these women (with a median urinary perchlorate concentration of 5 μ g/liter in the Turin cohort and 2 μ g/liter in the Cardiff cohort), no associations between urine perchlorate concentrations and serum TSH or free T4 in the individual euthyroid or hypothyroid/hypothyroxinemic cohorts were found.

The data assessing the effect of maternal perchlorate exposure in neonates and children and thyroid function remain unclear (Leung et al., 2010).

Decreased iodine intake can decrease TH production, and therefore exposure to perchlorate might be particularly detrimental in iodine-deficient individuals (Leung et al. 2010). Moreover, biologically based dose-response modeling of the relationships among iodide status (e.g., dietary iodine levels), perchlorate dose, and TH production in pregnant women has shown that iodide intake has a profound effect on the likelihood that exposure to goitrogens will produce hypothyroxinemia (Lewandowski et al. 2015).

Consequences of TH deficiency depend on the developmental timing of the deficiency (Zoeller and Rovet, 2004). For instance, if the TH deficiency occurs during early pregnancy, offspring show problems in visual attention, visual processing and gross motor skills, while if it occurs later, offspring may show subnormal visual and visuospatial skills, slower response speeds and motor deficits. If TH insufficiency occurs after birth, language and memory skills are most predominantly affected (Zoeller and Rovet, 2004). Altogether these studies indicate that factors, such as age, gender, developmental stage, and iodide status can affect the impact of perchlorate and other NIS inhibitors. All these variables should be taken into account to explain possible inconsistencies in study findings.

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Relationship: 872: Thyroidal Iodide, Decreased leads to TH synthesis, Decreased (<https://aopwiki.org/relationships/872>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	adjacent	High	High
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

Empirical evidence comes from *in vivo* studies in rats (Wu F et al., 2012; Davidson et al., 1978), *in vitro* studies using thyroid follicular rat cells (Spitzweg et al., 1999; Sue et al., 2012) and porcine thyroid follicles (Sugawara et al., 1999), and human epidemiological studies (Steinmaus et al., 2016b; Horton et al., 2015; Brechner et al., 2000)

Key Event Relationship Description

Thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3) are synthesized in the thyroid gland in the presence of functional NIS and thyroid peroxidase (TPO) as iodinated thyroglobulin (Tg), and stored in the colloid of thyroid follicles. NIS is a membrane bound glycoprotein whose main physiological function is to transport one iodide ion along with two sodium ions across the basolateral membrane of thyroid follicular cells. Extensive studies on NIS protein have identified 14 different mutations and each one of them is related to Iodine Transport Deficiencies (ITD) (Spitzweg and Morris, 2010). Once inside the follicular cells, the iodide diffuses to the apical membrane, where it is metabolically oxidized through the action of TPO to iodinium (I⁺), which in turn iodinates tyrosine residues of the Tg proteins in the follicle colloid. Therefore, NIS is essential for the synthesis of thyroid hormones (T3 and T4). TPO is a heme-containing apical membrane protein within the follicular lumen of thyrocytes that acts as

the enzymatic catalyst for TH synthesis (Taurog, 2005). Propylthiouracil (PTU) and methimazole (MMI), are thioureylene drugs that are known to inhibit the ability of TPO to: a) activate iodine and transfer it to thyroglobulin (Tg) (Davidson et al., 1978) and, b) couple thyroglobulin (Tg)-bound iodotyrosyls to produce Tg-bound T3 and T4 (Taurog, 2005). PTU and MMI have been found to decrease also the expression of NIS mRNA and consequently iodide accumulation, as shown in FRTL-5 cells (Spitzweg et al. 1999).

Other compounds, such as tricosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and bisphenol A (BPA) have been reported to decrease thyroid hormone (TH) levels by inducing an inhibition of NIS-mediated iodide uptake and altering the expression of genes involved in TH synthesis in rat thyroid follicular FRTL-5 cells, and on the activity of thyroid peroxidase (TPO), using rat thyroid microsomes (Wu Y et al. 2016).

Perchlorate, thiocyanate, nitrate, and iodide, which are competitive inhibitors of iodide uptake, have been shown to inhibit radioactive iodide uptake by NIS (Tonacchera et al. 2004), consequentially resulting in inhibition of TH synthesis. In particular, perchlorate blocks iodide uptake into the thyroid through NIS inhibition and decreases the production of TH (Steinmaus, 2016a). More recent evidence also suggests that young children, pregnant women, foetuses, and people co-exposed to similarly acting agents may be especially susceptible to perchlorate-induced toxicity (Steinmaus et al., 2016b).

Concern about environmental perchlorate exposure is focused on its inhibition of iodide uptake into the thyroid (MIE). Decreased iodine intake may decrease thyroid hormone production. Perchlorate exposure, therefore, might be particularly detrimental in iodine-deficient individuals. Median urinary iodine levels are used instead and reflect dietary iodine sufficiency across populations (International Council for the Control of Iodine Deficiency Disorders (ICCID); available from: www.iccidd.org (<http://www.iccidd.org>)). According to ICCIDD report iodine deficiency continues to be an important global public health issue, with an estimated 2.2 million people (38% of the world's population) living in iodine-deficient areas. In 1990, the United Nations World Summit for Children set forth the goal of eliminating iodine deficiency worldwide (UNICEF World Summit for Children. Available from: <http://www.unicef.org/wsc/declare.htm> (<http://www.unicef.org/wsc/declare.htm>); 1990). Considerable progress has been achieved by programmes of universal salt iodisation (USI) in various countries, in line with the recommendations of the World Health Organization (WHO) (WHO, UNICEF, ICCIDD. A guide for programme managers. World Health Organization; Geneva: 2007. Assessment of the iodine deficiency disorders and monitoring their elimination.WHO/NHD/01.1). However, many countries remain iodine deficient (de Benoist et al., 2013; Lazarus and Delange, 2004). In the U.S., data from large population studies have shown that median urinary iodine levels decreased by approximately 50% between the early 1970s and the early 1990s, although the population overall remained iodine sufficient (Hollowell et al., 1998). Subsequent studies have shown that this decrease has stabilised (Caldwell et al., 2005). The WHO still considers iodine deficiency, which leads to hypothyroidism, the single most important preventable cause of brain damage worldwide (WHO/UNICEF/ICCID, 2007). The most vulnerable groups are pregnant and lactating women and their developing fetuses and neonates, given the crucial importance of iodine to ensure adequate levels of thyroid hormones for brain maturation. Iodine deficiency in pregnancy is a prevailing problem not only in developing countries, but also in western industrialized nations and other countries classified as free of iodine deficiency, and solution may be found in dietary changes (Moog et al., 2017).

Evidence Supporting this KER

Biological Plausibility

The association between these two KEs is strong, and supported by *in vitro*, *in vivo* and epidemiological studies. Blocking iodide uptake into the thyroid follicular cells as a consequence of NIS inhibition or functional impairment, leads to reduced TH synthesis. Compounds that have been shown to inhibit NIS function (e.g., perchlorate, thiocyanate, nitrate, and iodide), has also been proven to decrease TH synthesis by inducing a downregulation of TPO gene expression and/or increase of TSH level, which are both indicative of a reduce TH biosynthesis. TSH receptor controls transcription and posttranslational modification of NIS (Dai et al., 1996). Stimulation of TSH receptor increases T3 and T4 production and secretion (Szkudlinski et al., 2002). NIS gene expression is suppressed by growth factors such as IGF-1 and TGF- β (the latter is induced by the BRAF-V600E oncogene), which prevent NIS to localize in the basolateral membrane (Riesco-Eizaguirre et al., 2009). The BRAF-V600E oncogene is also associated with downregulation TSH receptor (Kleiman et al. 2013). Altogether these studies support the association between NIS inhibition-induced decreased iodide uptake (KE up) and reduced TH synthesis (KE down).

Empirical Evidence

Several *in vitro* and epidemiological studies have shown that iodide uptake blockade occurring as a consequence of NIS (and TPO) inhibition leads to reduced TH synthesis:

- **Spitzweg et al., 1999:** In this *in vitro* study, a 48 hr treatment of FRTL-5 cells with MMI (100 μ M), PTU (100 μ M), and potassium iodide (40 μ M) induced ~ 50% decrease of NIS mRNA steady-state levels. Incubation with MMI and PTU resulted in a 20% and 25% decrease of iodide accumulation, respectively, whereas potassium iodide suppressed iodide accumulation by approximately 50%.
- **Wu Y et al., 2016:** This *in vitro* study showed that tricosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and bisphenol A (BPA) induced a concentration-dependent inhibition of NIS-mediated iodide uptake. Moreover, tricosan or triclocarban did not affect the expression of genes involved in TH synthesis (Slc5a5, TPO, and Tgo) or thyroid transcription factors (Pax8, Foxe1, and Nkx2-1). BDE-47 decreased the level of TPO, while BPA altered the expression of all six genes, as shown in rat thyroid follicular FRTL-5 cells. At the same time, tricosan and triclocarban also inhibited the activity of TPO at 166 and >300 μ M, respectively.
- **Steinmaus et al., 2016b:** In 1,880 pregnant women from San Diego County, California, during 2000–2003, it has been found that the presence of high level of perchlorate, thiocyanate, nitrate, and iodide in water supply induced a decrease of total thyroxine (T4) [regression coefficient (β) = -0.70; 95% CI: -1.06, -0.34], a decrease of free thyroxine (fT4) (β = -0.053; 95% CI: -0.092, -0.013), and an increase of thyroid-stimulating hormone (TSH), all indicators of reduced TH synthesis.
- **Horton et al., 2015:** in this study TSH levels measured in blood samples of 284 pregnant women at 12 (\pm 2.8) weeks gestation were found to positively correlate with the levels of urinary concentrations of perchlorate, nitrate and thiocyanate (NIS inhibitors), but perchlorate had the largest weight in the index, indicating the largest contribution to the weighted quantile sum regression. This indicates a perchlorate-dependent alteration of maternal thyroid function, through NIS inhibition.
- **Brechner et al., 2000:** Median newborn TSH levels in a city where drinking water supply was perchlorate-contaminated (from the Colorado River below Lake Mead) were significantly higher than those in a city totally supplied with non-perchlorate-contaminated drinking water, even after adjusting for factors known or suspected to elevate newborn TSH levels.
- **Charatcharoenwittaya et al. 2014:** this cross-sectional epidemiological study conducted in 200 pregnant Thai women with a gestational age of 14 weeks or less, showed that low-level exposure to perchlorate (i.e., 1.9 μ g/L of urinary perchlorate) was positively associated with TSH and negatively associated with free T4 using multivariate analyses in first-trimester pregnant women. Low thiocyanate urinary levels (510.5 μ g/L) were also positively associated with TSH in a subgroup of pregnant women with low iodine excretion (less than 100 μ g/L).

Several other studies have proven that NIS inhibitors lead to a decrease of thyroidal iodide uptake (Jones et al., 1996; Tonacchera et al., 2004; De Groef et al., 2006; Waltz et al., 2010), leading to a reduction of TH synthesis.

Uncertainties and Inconsistencies

Some studies have highlighted contradictory results in relation to response to chemicals. For instance, PTU and MMI have been shown to inhibit the activity of TPO in rats (Davidson et al., 1978), while inducing an increase of cellular TPO activity and TPO mRNA in cultured porcine thyroid follicles (Sugawara et al., 1999). PTU was also found to increase NIS gene expression, and the accumulation of 125 I, as shown in *in rat thyroid FRTL-5 cells*, while MMI had no effect (Sue et al., 2012).

Moreover, despite the well described effects of perchlorate, thiocyanate, nitrate, and iodide on iodide uptake into the thyroid, occupational and clinical dosing studies have not identified clear adverse effects, particularly in the case of perchlorate (Tarone et al. 2010). For instance, a longitudinal epidemiologic Chilean study found that there were no increases of thyroglobulin (Tg) or thyrotropin (TSH) levels, and no decreases of free T4 levels among either women during early pregnancy, late pregnancy, or the neonates at birth related to perchlorate in drinking water, suggesting that perchlorate in drinking water at 114 microg/L did not cause changes in neonatal thyroid function or fetal growth retardation (Téllez Téllez et al., 2005). Similarly, no associations between urine perchlorate concentrations and serum TSH or free T4 were found in individual euthyroid or hypothyroid/hypothyroxinemic cohorts of 261 hypothyroid/hypothyroxinemic and 526 euthyroid women from Turin and 374 hypothyroid/hypothyroxinemic and 480 euthyroid women from Cardiff (Pearce et al., 2010), suggesting that log perchlorate may not be a predictor of serum free T4 or TSH. However, it should be considered that these studies may be limited by short study durations, and the inclusion of mostly healthy adults (Steinmaus, 2016b).

Charnley's (2008) review examines several studies pointing out a number of inconsistent conclusions regarding link between TH serum levels, urinary iodine concentrations, and environmental perchlorate exposure (Charnley et al. 2008). For instance, no correlations were found between TH serum levels and urinary iodine concentrations among women exposed to perchlorate participating in the 2000–2001 National Health and Nutrition Examination Survey (NHANES). Available evidence does not support a causal relationship between changes in TH levels and current environmental levels of perchlorate exposure, but does support the conclusion that the US EPA's reference dose (RfD) for perchlorate is conservatively health-protective. However, potential perchlorate risks are unlikely to be distinguishable from the ubiquitous background of naturally occurring substances present at much higher exposures that can affect the thyroid via the same biological mode of action as perchlorate, such as nitrate and thiocyanate. Therefore, risk management approaches that account for both aggregate and cumulative exposures and that consider the larger public health context in which exposures are occurring are desirable.

In a cross-sectional analysis, McMullen et al. (2017) evaluated the exposure to perchlorate, thiocyanate, and nitrate (NIS inhibitors) could be a factor of age and sex. These results indicate that adolescent boys and girls represent the most vulnerable subpopulations to NIS symporter inhibitors. Therefore, discrepancies in results described in epidemiological studies may be due to difference in age of study participants.

Apart from age, relative source contribution of perchlorate exposure plays an important role in determining a significant reduction of serum TH levels. For instance, Lumen and George (2017) showed that there was no significant difference in geometric mean estimates of free T4 when perchlorate exposure from food only was compared to no perchlorate exposure in pregnant women. The reduction in maternal free T4 levels reached statistical significance when an added contribution from drinking water was assumed in addition to the 90th percentile of food intake for pregnant women. In particular, a daily intake of 0.45- 0.50 μ g/kg/day of perchlorate was necessary to produce results that were significantly different than those obtained from no perchlorate exposure. The authors comment that 'these modelling results can explain why findings from observational studies present inconsistent outcomes regarding the relationship between perchlorate exposure and thyroid hormone levels'.

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Relationship: 305: TH synthesis, Decreased leads to T4 in serum, Decreased (<https://aopwiki.org/relationships/305>)
AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/42)	adjacent	High	Moderate
XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/65)	adjacent	High	Moderate
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	adjacent	High	High
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	High	Moderate
Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/159)	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability			
Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	<i>Mus musculus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Xenopus laevis	<i>Xenopus laevis</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8355)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

While a majority of the empirical evidence comes from work with laboratory rodents, there is a large amount of supporting data from humans (with anti-hyperthyroidism drugs including propylthiouracil and methimazole), some amphibian species (e.g., frog), and some avian species (e.g., chicken). The following are samples from a large literature that supports this concept: Cooper et al. (1982; 1983); Hornung et al. (2010); Van Herck et al. (2013); Paul et al. (2013); Alexander et al. (2017).

Key Event Relationship Description

Thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3) are synthesized by NIS and TPO in the thyroid gland as iodinated thyroglobulin (Tg) and stored in the colloid of thyroid follicles. Secretion from the follicle into serum is a multi-step process. The first involves thyroid stimulating hormone (TSH) stimulation of the separation of the peptide linkage between Tg and TH. The next steps involve endocytosis of colloid, fusion of the endosome with the basolateral membrane of the thyrocyte, and finally release of TH into blood. More detailed descriptions of this process can be found in reviews by Braverman and Utiger (2012) and Zoeller et al. (2007).

Evidence Supporting this KER

The weight of evidence linking these two KEs of decreased TH synthesis and decreased T4 in serum is strong. It is commonly accepted dogma that decreased synthesis in the thyroid gland will result in decreased circulating TH (serum T4).

Biological Plausibility

The biological relationship between two KEs in this KER is well understood and documented fact within the scientific community.

Empirical Evidence

It is widely accepted that TPO inhibition leads to declines in serum T4 levels in adult mammals. This is due to the fact that the sole source for circulating T4 derives from hormone synthesis in the thyroid gland. Indeed, it has been known for decades that insufficient dietary iodine will lead to decreased serum TH concentrations due to inadequate synthesis. Strong qualitative and quantitative relationships exist between reduced TH synthesis and reduced serum T4 (Ekerot et al., 2013; Degon et al., 2008; Cooper et al., 1982; 1983; Leonard et al., 2016; Zoeller and Tan, 2007). There is more limited evidence supporting the relationship between decreased TH synthesis and lowered circulating hormone levels during development. Lu and Anderson (1994) followed the time course of TH synthesis, measured as thyroxine secretion rate, in non-treated pregnant rats and correlated it with serum T4 levels. More recently, modeling of TH in the rat fetus demonstrates the quantitative relationship between TH synthesis and serum T4 concentrations (Hassan et al., 2017). Furthermore, a wide variety of drugs and chemicals that inhibit TPO are known to result in decreased release of TH from the thyroid gland, as well as decreased circulating TH concentrations. This is evidenced by a very large number of studies that employed a wide variety of techniques, including thyroid gland explant cultures, tracing organification of 131-I and *in vivo* treatment of a variety of animal species with known TPO inhibitors (King and May, 1984; Atterwill et al., 1990; Brown et al., 1986; Brucker-Davis, 1998; Hornung et al., 2010; Hurley et al., 1998; Kohrle, 2008).

Temporal Evidence: The temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). There are currently no studies that measured both TPO synthesis and TH production during development. However, the impact decreased TH synthesis on serum hormones is similar across all ages. Good evidence for the temporal relationship comes from thyroid system modeling of the impacts of iodine deficiency and NIS inhibition (e.g., Degon et al., 2008; Fisher et al., 2013). In addition, recovery experiments have demonstrated that serum thyroid hormones recovered in athyroid mice following grafting of *in-vitro* derived follicles (Antonica et al., 2012).

Dose-response Evidence: Dose-response data is lacking from studies that include concurrent measures of both TH synthesis and serum TH concentrations. However, data is available demonstrating correlations between thyroidal TH and serum TH concentrations during gestation and lactation during development (Gilbert et al., 2013). This data was used to develop a rat quantitative biologically-based dose-response model for iodine deficiency (Fisher et al., 2013).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. The first uncertainty stems from the paucity of data for quantitative modeling of the relationship between the degree of synthesis decrease and resulting changes in circulating T4 concentrations. In addition, most of the data supporting this KER comes from inhibition of TPO, and there are a number of other processes (e.g., endocytosis, lysosomal fusion, basolateral fusion and release) that are not as well studied.

Quantitative Understanding of the Linkage**Response-response relationship**

Fisher et al. (2013) published a quantitative biologically-based dose-response model for iodine deficiency in the rat. This model provides quantitative relationships for thyroidal T4 synthesis (iodine organification) and predictions of serum T4 concentrations in developing rats. There are other computational models that include thyroid hormone synthesis. Ekerot et al. (2012) modeled TPO, T3, T4 and TSH in dogs and humans based on exposure to myeloperoxidase inhibitors that also inhibit TPO. This model was recently adapted for rat (Leonard et al., 2016) and Hassan et al (2017) have extended it to include the pregnant rat dam in response to TPO inhibition induced by PTU. While the original model predicted serum TH and TSH levels as a function of oral dose, it was not used to explicitly predict the relationship between serum hormones and TPO inhibition, or thyroidal hormone synthesis. Leonard et al. (2016) recently incorporated TPO inhibition into the model. Degon et al (2008) developed a human thyroid model that includes TPO, but does not make quantitative prediction of organification changes due to inhibition of the TPO enzyme.

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Relationship: 312: T4 in serum, Decreased leads to T4 in neuronal tissue, Decreased (<https://aopwiki.org/relationships/312>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/42)	adjacent	Moderate	Moderate
XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/65)	adjacent	Moderate	Low
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (https://aopwiki.org/aops/134)	adjacent	High	Low
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High
All life stages	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

The majority of the information on this KER comes from in vivo studies with rodents (mainly MCT8 knock-out mice and thyroidectomized rats) and histopathological analyses of human brain tissues derived from patients affected by AHDS (Allan-Herndon-Dudley syndrome). The evolutionary conservation of the transport of TH from circulation to the developing brain suggests, with some uncertainty, that this KER is also applicable to other mammalian species.

Key Event Relationship Description

In mammals, thyroxine (T4) in brain tissue is derived almost entirely from the circulating pool of T4 in blood. Transfer of free T4 (and to a lesser extent, T3) from serum binding proteins (thyroid binding globulin (TBG), transthyretin (TTR) and albumin; see McLean et al., 2017, for a recent review) into the brain requires transport across the blood brain barrier (BBB) and /or indirect transport from the cerebral spinal fluid (CSF) into the brain through the blood-CSF-barrier. The blood vessels in rodents and humans expresses the main T4 transporter, MCT8, (Roberts et al. 2008), as does the choroid plexus which also expresses TTR and secretes the protein into the CSF (Alshehri et al. 2015).

T4 entering the brain through the BBB is taken up into astrocytes via cell membrane iodothyronine transporters (e.g., organic anion-transporting polypeptides OATP), monocarboxylate transporter 8 (MCT8) (Visser et al., 2011). In astrocytes, T4 is then deiodinated by Type II deiodinase to triiodothyronine (T3) (St Germain and Galton, 1997), which is then transported via other iodothyronine transporters (MCT8) into neurons (Visser et al., 2011). While some circulating T3 may be taken up into brain tissue directly from blood (Dratman et al., 1991), the majority of neuronal T3 comes from deiodination of T4 in astrocytes. Decreases in circulating T4 will eventually result in decreased brain T3 tissue concentrations. It is also known that Type II deiodinase can be up-regulated in response to decreased T4 concentrations to maintain tissue concentrations of T3 (Pedraza et al., 2007; Lavado-Autric et al., 2013; Morse et al., 1986), except in tanyocytes of the paraventricular nucleus (Fekete and Lechan, 2014).

Evidence Supporting this KER

The weight of evidence linking reductions in circulating serum TH and reduced brain concentrations of TH is moderate. Many studies support this basic linkage. However, there are compensatory mechanisms (e.g., upregulation of deiodinases, transporters) that may alter the relationship between hormones in the periphery and hormone concentrations in the brain. There is limited information available on the quantitative relationship between circulating levels of TH, these compensatory processes, and neuronal T4 concentrations, especially during development. Furthermore, in certain conditions, such as iodine deficiency, the decreases in circulating hormone might have greater impacts on tissue levels of TH (see for instance, Escobar del Rey, et al., 1989).

Biological Plausibility

The biological relationship between these two KEs is strong as it is well accepted dogma within the scientific community. There is no doubt that decreased circulating T4 leads to declines in tissue concentrations of T4 and T3 in a variety of tissues, including brain. However, compensatory mechanisms (e.g., increased expression of Type 2 deiodinase) may differ during different lifestages and across different tissues, especially in different brain regions. Similarly, the degree to which serum TH must drop to overwhelm these compensatory responses has not been established.

Empirical Evidence

Several studies have shown that tissue levels, including brain, of TH are proportional to serum hormone level (Oppenheimer, 1983; Morreale de Escobar et al., 1987; 1990; Calvo et al., 1992; Porterfield and Hendrich, 1992, 1993; Broedel et al., 2003). In thyroidectomized rats, brain concentrations of T4 were decreased and Type II deiodinase (DII) activity was increased. Both brain T3 and T4 as well as DII activity returned to normal following infusion of T4 (Escobar-Morreale et al., 1995; 1997). Animals treated with PTU, MMI, or iodine deficiency during development demonstrate both lower serum and lower brain TH concentrations (Escobar-Morreale et al 1995; 1997; Taylor et al., 2008; Bastian et al., 2012; 2014; Gilbert et al., 2013). Compared to the wildtype, a mouse MCT8 knockout model has been shown to have decreased plasma T4, decreased uptake of T4 into the brain, and decreased brain T3 concentrations, as well as increased cortical deiodinase Type 2 activity and increased plasma T3 concentrations (Mayerl et al., 2014; Barez-Lopez et al., 2016).

Temporal Evidence: The temporal relationship between serum T4 and T4 in growing neuronal tissue described in this KER is developmental (Seed et al., 2005). While all brain regions will be impacted by changes in serum hormones, brain concentrations will be a function of development stage and brain region. Data are available from thyroid hormone replacement studies that demonstrate recovery of fetal brain T3 and T4 levels (following low iodine diets or MMI exposure) to control levels after maternal thyroid hormone replacement or iodine supplementation (e.g., Calvo et al., 1990; Obregon et al., 1991). For example, Calvo et al. (1990) carried out a detailed study of the effects of TPO inhibition on serum and tissues levels of TH in gestating rats. Clear dose-dependent effects of T4 replacement, but not T3 replacement were seen in all maternal tissues. However, for fetal tissues, neither T4 nor T3, at any dose, could completely restore tissue TH levels to control levels.

Dose-Response Evidence: There is good evidence, albeit from a limited number of studies of the correlative relationship between circulating thyroid hormone concentrations and brain tissue concentrations during fetal and early postnatal development following maternal iodine deficient diets or chemical treatments that depress serum THs (c.f., Calvo et al., 1990; Obregon et al., 1991; Morse et al., 1996).

Uncertainties and Inconsistencies

The fact that decreased serum TH results in lower brain TH concentrations is well accepted. However, the ability of the developing brain to compensate for insufficiencies in serum TH has not been well studied. Limited data is available that demonstrates that changes in local deiodination in the developing brain can compensate for chemical-induced alterations in TH concentrations (e.g., Calvo et al., 1990; Morse et al., 1996; Sharlin et al., 2010). And, there are likely different quantitative relationships between these two KEs depending on the compensatory ability based on both developmental stage and specific brain region (Sharlin et al., 2010). For these reasons, the empirical support for this linkage is rated as moderate

The role of cellular transporters represents an additional uncertainty. In addition, future work on cellular transport mechanisms and deiodinase activity is likely to inform addition of new KEs and KERs between serum and brain T4.

Quantitative Understanding of the Linkage

Response-response relationship

While it is well established that decreased in serum TH levels result in decreased brain TH concentrations, particularly fetal brain concentrations, a major gap is the lack of empirical data that allow direct quantification of this relationship (Hassan et al., 2018). Recently, serum TH and brain TH were measured in fetal cortex and postnatal day 14 offspring following graded degrees of hypothyroidism induced by PTU (O'Shaughnessy et al., 2018). Results showed that brain levels TH levels at both ages were quantitatively related to serum T4 levels. Additional dose-response information is necessary to confirm these findings, and standardization of analysis for the measurements in these distinct matrices is crucial to allow comparisons to be made between independent experiments.

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Relationship: 444: T4 in neuronal tissue, Decreased leads to BDNF, Reduced (<https://aopwiki.org/relationships/444>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	High
Adult	Moderate
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

The connection between TH levels and BDNF expression has been studied only in rodent models up to date (see above studies).

Key Event Relationship Description

It is widely accepted that the thyroid hormones (TH) have a prominent role in the development and function of the Central Nervous System (CNS) and their action has been closely linked to the cognitive function because of their importance in the neocortical development (Gilbert et al., 2012). During the early cortical network development TH has been shown to influence the number of cholinergic neurons and the degree of innervation of hippocampal CA3 and CA1 regions (Oh et al., 1991; Thompson and Potter 2000), and to regulate the morphology and function of GABAergic neurons (Westerholz et al., 2010).

One of the mediators of this regulation has been suggested to be the brain derived neurotrophic factor (BDNF), whose role in brain development and function has been very well-documented (Binder and Scharfman, 2004) and which function has been associated with TH levels in the brain (Gilbert and Lasley, 2013). Several studies have shown that TH can regulate BDNF expression in the brain (Koibuchi et al., 1999; Koibuchi and Chin, 2000; Sui and Li, 2010), with the subsequent neurodevelopmental consequences.

In view of the above evidence, it has been shown that the thyroid insufficiency (lower TH levels) results in reduction of BDNF levels (mRNA or protein) in the developmental brain.

Evidence Supporting this KER

Biological Plausibility

The importance of thyroid hormones (TH) in brain development has been recognised and investigated for many decades (Bernal, 2011). Several human studies have shown that low levels of circulating maternal TH, even in the modest degree, can lead to neurophysiological deficits in the offspring, including learning and memory deficits, or even cretinism in most severe cases (Zoeller and Rovet, 2004; Henrichs et al., 2010). The levels of serum TH at birth are not always informative, as most of the neurological deficits are present despite the normal thyroid status of the newborn. That means that the cause of these impairments is rooted in the early stages of the neuronal development during the gestational period. The nature and the temporal occurrence of these defects suggest that TH may exert their effects through the neurotrophins, as they are the main regulators of neuronal system development (Lu and Figurov, 1997). Among them, BDNF represents the prime candidate because of its critical role in CNS development and its ability to regulate synaptic transmission, dendritic structure and synaptic plasticity in adulthood (Binder and Scharfman, 2004). Additionally, hippocampus and neocortex are two of the regions characterized by the highest BDNF expression (Kawamoto et al., 1996), and are also key brain areas for learning and memory functions. Indeed, it has been shown that the thyroid insufficiency (lower TH levels) results in reduction of BDNF levels (mRNA or protein) in the developing brain, and the most likely affected brain regions are the hippocampus and cortex (Koromilas et al., 2010; Shafiee et al., 2016). The hippocampus direct and indirect interactions with the THs provide crucial information on the neurobiological basis of the hypothyroidism-induced mental retardation and neurobehavioral dysfunction. TH deficiency during the foetal and/or the neonatal period produces deleterious effects for neural growth and development (such as reduced synaptic connectivity, delayed myelination, disturbed neuronal migration, deranged axonal projections, decreased synaptogenesis and alterations in neurotransmitters' levels), possibly through decreased BDNF levels (Koromilas et al., 2010; Shafiee et al., 2016).

Empirical Evidence

The empirical support for this direct KER is moderate. There are a limited number of studies investigating TH concentrations in the brain and BDNF levels. This is due to the fact that TH is difficult to measure in the brain and TH-induced changes in BDNF expression (mRNA or protein levels) can be subtle.

For the current AOP the temporal nature of this KER is described in the context of different developmental stages (Seed et al., 2005). The impact of brain TH concentrations on regulation of TH receptor (TR) regulated genes is age-dependent for a number of genes critical for normal hippocampal development. It is widely accepted that different genes, including BDNF, are downregulated under conditions of TH insufficiency (Pathak et al., 2011). TH supplementation has been shown to reverse some of the effects on gene expression (Liu et al., 2010; Pathak et al., 2011), including also BDNF expression (Wang et al., 2012).

Many *in vivo* studies have focused on the determination of the relationship between TH-mediated effects and BDNF expression in the brain. The majority of the work has been performed by evaluating the effects of TH insufficiency on BDNF developmental expression profile. The results, despite some differences, are showing a trend toward BDNF down-regulation.

Reductions in BDNF mRNA and protein were observed in hypothyroid rat models, created by exposing the animals to the TPO inhibitors methimazole (MMI) (Sinha et al., 2009) or propylthiouracil (PTU) (Neveu and Arenas, 1996; Lasley and Gilbert, 2011), and perchlorate (NIS inhibitor) (Koibuchi et al., 1999; 2001). These studies supported direct associations between lower level of TH and lower BDNF expression in the developmental cerebellum, hippocampus and cortex. The dose-response relationship could not be evaluated in these studies, as they were conducted in conditions of severe maternal hypothyroidism, namely after exposure to very high doses of the chemicals.

Additionally, in more complex models of maternal hypothyroidism in rats a reduction of hippocampal and cortex BDNF expression was observed (Wang et al., 2012; Liu et al., 2010). In these latter cases, hypothyroidism was developed via thyroidecomy, and T4 supplementation was performed at specific stages during gestation.

Indirect evidences supporting this KER:

- Koibuchi et al., 2001: in this *in vivo* study, newborn mice were rendered hypothyroid by administering MMI (TPO inhibitor) and perchlorate (NIS inhibitor) in drinking water to their mothers. Neurotrophin-3 (NT-3) and BDNF gene expression was depressed in the perinatal hypothyroid cerebellum. Since TH levels in the brain were not measured, the same study was also included in the indirect KER "TH synthesis decrease leads to BDNF reduction".

- Morte et al., 2010: in this *in vivo* study, maternal and fetal hypothyroidism was induced by maternal thyroidectomy (Tx) followed by the antithyroid drug MMI (TPO inhibitor) treatment. Tx dams treated with MMI showed increased TSH (~10 fold increase) and decreased serum T4 (~95%) and serum T3 (~90%). Whilst fetuses from Tx rats had normal serum TSH and cerebral cortex T4 and T3, pups born from dams treated with MMI showed increase of TSH (~8 fold), decreased cortex T4 (~75%) and cortex T3 (~95%). Additionally, analysis of gene expression in the fetal cerebral cortex showed a reduction

of *Camk4* (Ca(2+) and calmodulin-activated kinase) gene expression (~70%) induced by maternal and fetal hypothyroidism. As *Camk4* during development is known to induce BDNF expression (Shieh et al., 1998), a reduction of cortical TH levels (leading to *Camk4* mRNA downregulation) may lead to reduction of BDNF expression and/or signaling; however, BDNF expression was not measured in Morte et al. 2010.

- **da Conceição et al., 2016:** in this *in vivo* study, thyroidectomized (i.e., hypothyroid) adult Wistar rats showed significant increase of serum TSH (~ 750% increase vs control rats), decrease of T4 (~ 80% decrease vs control) and T3 serum levels (~ 45% decrease vs control), together with a reduced hippocampal expression of MCT8 (~ 83% decrease vs control rats), TH receptor alfa (TR α 1) (~ 77% decrease vs control), deiodinase type 2 (DIO2) (~ 90% decrease vs control), and BDNF mRNA expression in hippocampus (~ 75% decrease vs control). The reduced gene expression of TR α 1 (expressed in nearly all neurons in the brain (Schwartz et al., 1992)), MCT8 and DIO2 (both essential for the regulation of glia-neuron cell interaction in TH metabolism in the brain (Remaud et al., 2014)), indicate the presence of low TH levels in the hippocampus (hippocampal hypothyroidism) in thyroidectomized rats. However, direct measures of TH (T3 or T4) brain levels were not assessed in this study.

- **Pathak et al., 2011:** in this *in vivo* study, the effect of maternal TH deficiency on neocortical development was investigated. Rat dams were exposed to MMI (TPO inhibitor) from GD6 until sacrifice. Decreased number and length of radial glia, loss of neuronal bipolarity, and impaired neuronal migration were recovered with early TH replacement (at E13-15). BDNF mRNA resulted downregulated (80% decrease at E14) while *trkB* expression was increased (2-fold) in hypothyroid fetuses at E14 stage. However, TH levels in the brain were not measured in this study.

Sui et al. 2010: in this *in vivo* study, T3, rT3 or vehicle were administered to young adult male rats either via systemic injection (i.e., IP) administration of a single dose of 30 μ g of T3/100 g body weight or the same dose of rT3 or the same volume of vehicle solution), or local brain infusion (i.e., intrahippocampal bilateral infusion (in the dorsal region) with 50 pmol T3, 50 pmol rT3 or vehicle (0.05% ethanol and saline solution), in 1 μ l). Data showed that T3 administration increased reelin (~ 3-fold increase relative to the vehicle group at 24 h following T3 IP injection, and ~ 7-fold increase relative to the vehicle group after 1 h following T3 intrahippocampal injection), total BDNF (~ 10-fold increase relative to the vehicle group at 24 h after IP injection, and ~ 6-fold increase relative to the vehicle group at 12 h after intrahippocampal injection), and exon-specific BDNF mRNA expression in the hippocampus (after T3 IP injection: BDNF exon I and II transcripts was ~ 50-fold higher compared to the vehicle levels, whereas exon IV and VI transcripts were ~ 10-fold and ~ 30-fold higher respectively; after T3 intrahippocampal injection: exon I and II: 3.5 to 4-fold; exon IV: ~ 7-fold; exon VI: ~ 12-fold relative to the vehicle, respectively at 2 h to 24 h (for exon I), 1 h and 6 h (for exon IV), and 12 h (for exon VI) after T3 infusion).

Conversely, administration of rT3 (inactive T3 isoform) through IP injection or intrahippocampal infusion did not significantly alter the hippocampal reelin or BDNF mRNA levels (two pathways critical for learning and memory processes regulation). Reelin protein levels resulted increased upon both IP injection (2-fold increase 24 h after injection) and intrahippocampal infusion of T3 (3-fold increase 4 h after infusion). Likewise, BDNF protein levels were upregulated after IP injection (~ 2.7-fold increase 24 h after injection) and intrahippocampal infusion (~ 2.5-fold increase 12 h after infusion) of T3. Analysis of transcriptional coactivators binding (e.g., cAMP response element binding protein-binding protein (CBP), and thyroid hormone receptor associated protein 220 (TRAP 220)) and RNA polymerase II (RNA Pol II) revealed specific patterns of associations between such transcription factors and reelin or BDNF upon T3 administration. This study suggests that hippocampal BDNF mRNA and protein expression is under T3 regulation.

- **Blanco et al. 2013:** in this *in vivo* study rat dams were exposed to 0, 1 and 2 mg/kg/day of BDE-99 from GD 6 to PND 21. Data showed that transmission of maternal accumulated BDE-99 through placenta and breast milk caused a decrease of serum levels of T3 (by 13 \pm 9% in the 2 mg/kg/day group), T4 (by 25 \pm 13% in the 2 mg/kg/day group) and free-T4 (by up to 17 \pm 9% in the 2 mg/kg/day group), causing downregulation of BDNF gene expression in the hippocampus of pups (by 32 \pm 14% in the 2 mg/kg/day group). On the contrary, the expression of other TRs isoforms did not change in both cortex and hippocampus. Moreover, BDE-99 produced a delay in the spatial learning task in the water maze test (i.e., longer latency in reaching the platform at the highest BDE-99 dose vs control group), and a dose-response anxiolytic effect as revealed by the open-field test.

- **Abdelhaffez and Hassan, 2013:** in this *in vivo* study rat dams were exposed to MMI (TPO inhibitor) to induce hypothyroidism. Pups showed a decrease of plasma free T3, free T4, and growth hormone (GH), whilst plasma TSH was significantly increased. BDNF level was significantly decreased in both the hippocampus and cerebellum of rat pups.

- **Shafee et al., 2016:** in this *in vivo* study rat dams were exposed to PTU (100 mg/L in drinking water) from embryonic day 6 to their PND 21. For 14 days (from PND 31 to 44), the rat pups were trained with either the mild treadmill exercise (TE) or the voluntary wheel exercise (VE). On PND 45-52, a water maze was used for testing their learning and memory ability. Hippocampal BDNF levels were assessed one day later. Data showed that pups exposed to PTU underwent a reduction of T4 levels (~ 70%) and an increase of TSH levels (~ 80%) at PND21. A reduction of hippocampal BDNF levels (~ 7-8% reduction comparing sedentary hypothyroid vs sedentary control pups) was observed in the treatment group. The conclusion of this quantitative study is that hypothyroidism during the foetal period and the early postnatal period is associated with the impairment of spatial learning and memory (e.g., ~ 55-60% increase of platform location latency in both sedentary hypothyroid male and female rats), and reduced hippocampal BDNF levels in both male and female rat offspring. Importantly, physical exercises (both VE and TE) significantly increased BDNF levels in both male and female hypothyroid animals (by ~ 2-3 percentage points) and improved learning and memory skills. Authors concluded that: "These findings suggest that the increase in BDNF levels following a period of physical activity in hypothyroid rat pups is an important mechanism by which exercise alleviates the learning and memory deficits induced by hypothyroidism".

- **Shi et al. 2017:** in this *in vivo* study rat dams were randomly treated with BDE-209 (100, 300, and 900 mg/kg body weight) or corn oil by gavage on gestational days 6 to 20. Blood was obtained through heart puncture for TH analysis in male rat offspring on PND 60. Data indicated that BDNF protein levels in the hippocampus decreased by 13% and 33% respectively in the 300 mg/kg and 900 mg/kg dose group. Total T4 levels and free T4 levels were significantly decreased in the BDE-209 treated-group (900 mg/kg, 300 mg/kg), and total T3 levels in 300 mg/kg group were also significantly decreased compared ctr (no significant difference was observed in 100 mg/kg group). In this study, decreased BDNF levels are well correlated with decrease of total and free T4 levels occurring upon exposure to BDE-209.

- **Mokhtari et al. 2017:** in this *in vivo* study rats underwent transient middle cerebral artery occlusion (tMCAo) to induce ischemic brain stroke. Rats were randomly divided in four groups: Co (control), Sh (sham), tMCAo and tMCAo + T3 (intracerebroventricular injection of T3 at 25 μ g/kg body administered 24 after reperfusion). T3 significantly improved the learning and memory compared with tMCAo group, as shown by Morris water maze test. Step-through latency significantly increased in the T3 group compared with tMCAo group. Moreover, BDNF mRNA and protein levels were decreased in the tMCAo compared with Co and Sh group (~ 15% decrease of protein and ~ 20% decrease of mRNA vs Co or Sh), and addition of T3 increased BDNF mRNA and proteins compared to Co, Sh and tMCAo groups (~ 94% increase of protein and ~ 750% increase of mRNA comparing tMCAo + T3 vs tMCAo). This study points out again that BDNF levels are under the control of T3.

- **Sabbaghzirani et al. 2017:** similar to previous study from Mokhtari et al. (2017), here cerebral ischemia was induced by MCAo in male Wistar rats; a group of rats was also injected with T3 (25 μ g/kg, IV injection) at 24 hours after ischemia. BDNF gene and protein levels (along with nestin and Sox2) were increased upon T3 treatment vs ischemic group. T3 treated rats also showed higher levels of serum T3 and T4, and lower levels of TSH vs ischemic group 4 days post ischemia induction. These data globally indicate that brain increased T3 levels increase BDNF expression and protein levels.

- **Kawahori et al. 2018:** in this *in vivo* study rat dams were administered with MMI (0.025% w/v) from 2 weeks prior to conception until delivery, which induced mild maternal hypothyroxinemia during pregnancy, comparing MMI and control offspring at day 28 and day 70 after birth. MMI-exposed pups showed an impaired learning capacity in the behavior tests. Hippocampal steady-state *Bdnf* exon IV (responsible for neural activity-dependent *Bdnf* gene expression) expression was lower in MMI group than in Ctr at day 28, while at day 70, hippocampal *Bdnf* exon IV expression at the basal level was comparable between the two groups. Additionally, persistent DNA hypermethylation was found in the promoter region of *Bdnf* exon IV in the hippocampus of MMI group vs ctr, which may be responsible for the decrease of *Bdnf* exon IV expression in the treated group.

Uncertainties and Inconsistencies

Hypothyroidism (i.e., induced by chemicals known to inhibit TPO or NIS, or by thyroidectomy, leading to low TH serum levels) is generally associated with lower levels of BDNF in brain tissues. As described in Conceição et al., 2016, thyroidectomized adult rats, apart from showing reduced TH levels, also presented reduced hippocampal gene expression of MCT8, TR α 1, DIO2 and BDNF, which support a link between hippocampal hypothyroidism and reduced BDNF levels.

However, despite the fact that many *in vivo* studies have shown a correlation between hypothyroidism and BDNF expression in the brain, there are no studies simultaneously measuring the levels of both TH and BDNF in the brain. Therefore, no clear consensus can be reached by the overall evaluation of the existing data. There are numerous conflicting studies showing no significant change in BDNF mRNA or protein levels under hypothyroid conditions (Alvarez-Dolado et al., 1994; Bastian et al., 2010; 2012; Royland et al., 2008; Lasley and Gilbert, 2011). However, the results of these studies cannot exclude the possibility of temporal- or region-specific decreased BDNF effects as a consequence of foetal hypothyroidism. A transient TH-dependent BDNF reduction in early postnatal life can be followed by a period of normal BDNF levels or, on the contrary, normal BDNF expression in the early developmental stages is not predictive of equally normal BDNF expression throughout development. Moreover, significant differences in study design, the assessed brain regions, the age and the method of assessment in the existing studies, further complicate result interpretation.

- In Alvarez-Dolado et al. 1994, hypothyroid rats showed decreased *trk* (BDNF receptor) mRNA levels in the striatum on PND 5, PND 15 and in adults, increase of p75LNGFR mRNA in hypothyroid cerebellum on PND 5 and PND 15, decrease of NGF mRNA in the cortex, hippocampus, and cerebellum of hypothyroid rats on neonatal hypothyroid rats on PND 15 and also after adult-onset hypothyroidism, whilst the relatively high expression of the two BDNF mRNAs did not change in any brain area.

- Bastian et al. (2010) assessed the effects Cu and Fe deficiencies on circulating and brain TH levels during development in pregnant rat dams rendered Cu deficient (CuD), Fe deficient (FeD), or TH deficient (by PTU treatment) from early gestation through weaning. Serum T4 and T3, and brain T3 levels were subsequently measured in PND 12 pups. Despite the remarkable decrease of serum TH and brain T3 induced by PTU treatment (and also by CuD and FeD), no significant changes of *Bdnf* IV mRNA levels were found. Authors commented that 'one explanation for this discrepancy is that many of the previous studies were performed using discrete brain regions, whereas this study was performed on whole-brain RNA'. Along the same line, in a follow up study, Bastian et al. (2012) could not find statistically significant reductions of *Bdnf* IV, *Bdnf* VI, and total *Bdnf* mRNA levels in hippocampus or cerebral cortex of Fe and TH deficient pups.

- Royland et al. (2008) assessed the effects of a PTU (TPO inhibitor) administration to pregnant rats from gestational day 6 until sacrifice of pups prior to weaning. However, PTU treatment did not change the expression of *Bdnf* at the mRNA level.

- In Lasley and Gilbert, 2011 study, different concentrations of PTU were administered to rat pregnant dams from gestational day 6 until weaning of the pups. Pups were sacrificed on PND 14, PND 21 and PND 100, analysis of TH serum levels was performed, along with analysis of hippocampal, cortical, and cerebellar levels of BDNF protein. While PTU caused a strong decrease of TH serum levels, no differences in BDNF protein were detected in the pre-weaning animals as a function of PTU exposure. On the contrary, dose-dependent decrease of BDNF levels emerged in adult males as a consequence of prenatal exposure despite the return to control TH levels. These findings reflect the potential for delayed impact of even modest TH reductions during critical periods of brain development on BDNF, a protein important for normal synaptic formation, as commented by the authors of this study.

It should also be considered that in severe models of TH deficiency, BDNF responsiveness to TH is regulated in a promoter-, age-, and brain region-specific fashion (as described by Anderson and Mariash, 2002), and even modest differences of these parameters in study design may explain inconsistencies in study results.

The absence of significant changes in BDNF levels in the above cited studies could be also due to different sensitivity of analytical tools, experimental design and statistical processing of the results.

While PTU (TPO inhibitor) has been shown to decrease serum TH levels and brain BDNF protein levels and mRNA expression in offspring born from PTU-treated rat dams (Shafiee et al. 2016; Chakraborty et al., 2012; Gilbert et al. 2016), in Cortés et al., 2012 study, treatment of adult male Sprague-Dawley rats with PTU induced an increase in the amount of BDNF mRNA in the hippocampus, while the content of TrkB, the receptor for BDNF, resulted reduced at the postsynaptic density (PSD) of the CA3 region compared with controls. Treated rats presented also thinner PSD than control rats, and a reduced content of NMDA_R subunits (NR1 and NR2A/B subunits) at the PSD in hypothyroid animals. While these data indicate differential effects elicited by PTU (i.e., TPO inhibition) on BDNF expression/regulation comparing the adult vs foetal brain, downregulation of TrkB receptors still leads to decrease signalling pathways regulated by BDNF.

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Relationship: 870: BDNF, Reduced leads to GABAergic interneurons, Decreased (<https://aopwiki.org/relationships/870>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

Empirical evidence comes from work with laboratory rodents (rats and mice). No data are available for other species.

Key Event Relationship Description

GABAergic interneurons are remarkably diverse and complex in nature and they are believed to play a key role in numerous neurodevelopmental processes (Southwell et al., 2014). Among them, those that express parvalbumin (PV) (marker of GABAergic interneurons) as their calcium-binding protein are the ones subjected to regulations by neurotrophins and BDNF specifically (Woo and Lu, 2006). These neurons do not express the BDNF protein but its functional receptor, TrkB (Cellerino et al., 1996; Marty et al., 1996; Gorba and Wahle, 1999). BDNF is released by the BDNF-producing neurons of the CNS and binds to TrkB of the GABA PV-interneurons, an interaction necessary for the subsequent developmental effects mediated by BDNF (Polleux et al., 2002; Jin et al., 2003; Rico et al., 2002; Aguado et al., 2003). BDNF promotes the morphological and neurochemical maturation of hippocampal and neocortical interneurons and promotes GABAergic synaptogenesis (Danglot et al., 2006 and Hu and Russek, 2008). BDNF also regulates the expression of the GABA-specific K⁺/Cl⁻ co-transporter, KCC2, which is responsible for switching of GABA action from excitatory to inhibitory, and consequently determines the nature of GABA-induced development of glutamatergic (excitatory) synapses (Wang and Kriegstein, 2009; Blaesse et al., 2009).

Evidence Supporting this KER

Biological Plausibility

Proper function of the Central Nervous System (CNS) results from the closely regulated development and function of the different neuronal subtypes and is driven by the overall balance between excitation and inhibition. In the cerebral cortex the synaptic inhibition is mediated by the GABAergic interneurons, which regulate also the neuronal developmental excitability and thereby the function and maturation of the neuronal networks (Voigt et al., 2001; Cherubini et al., 2011).

Many trophic factors are implicated in the regulation of these processes but among them BDNF stands out as the prime candidate due to its effects on interneuron development (Palizvan et al., 2004; Patz et al., 2004; Woo and Lu, 2006; Huang et al., 2007; Huang, 2009). Exogenous application of BDNF in developing neocortical and hippocampal GABAergic interneurons has demonstrated an enhanced dendrite elongation and branching in cultures (Jin et al., 2003; Vicario-Abejon et al., 1998). Interneuron differentiation was also affected by endogenous BDNF, as the length and branching of GABAergic interneurons (GFP-positive (i.e., BDNF^{+/+})), was promoted only when they were innervated by BDNF-releasing interneurons (Kohara et al., 2003). Due to these dendrite effects of BDNF on GABAergic interneurons, this neurotrophin was suggested to promote also the formation of inhibitory synapses, which was further supported by several *in vitro* studies. Exogenous application of BDNF significantly increased the number of functional synapses in culture (Vicario-Abejon et al., 1998; Marty et al., 2000), while blocking BDNF with antibodies greatly reduced the formation of inhibitory synapses (Seil and Drake-Baumann, 2000). Similar results were observed *in vivo* in transgenic mice with deleted TrkB gene in cerebellar precursors, in which TrkB receptor was found to be the prerequisite for inhibitory synapses formation (Rico et al., 2002). Additionally, BDNF was reported to elicit presynaptic changes in GABAergic interneurons, as several presynaptic proteins were up-regulated after BDNF application (Yamada et al., 2002; Berghuis et al., 2004). A significant increase of GABA receptor density was observed in cultured hippocampus-derived neurons after treatment with BDNF (Yamada et al., 2002).

BDNF is also a potent regulator of spontaneous neuronal activity (Aguado et al., 2003; Carmona et al., 2006), a major milestone of the developing hippocampus and an important feature of the CNS. Further supporting studies have shown that it has the ability to depolarize cortical neurons in culture (Kafitz et al., 1999), an effect which has been linked to the developmentally regulated spontaneous network activity (Feller, 1999; O'Donovan, 1999).

The spontaneous neuronal activity early in development is also closely related to Cl⁻ homeostasis, which is developmentally regulated by KCC2, the main K⁺/Cl⁻ co-transporter in the brain (Rivera et al., 1999). Because neuronal expression of KCC2 is low during early development, the intracellular [Cl⁻] cannot be extruded leading to the depolarizing effect of GABA during this period (Ben-Ari et al., 2004). Taking these under consideration, it was demonstrated that the effects of BDNF on neuronal activity was mediated by the KCC2 regulation, as observed in several *in vitro* and *in vivo* studies (Ludwig et al., 2011a and 2011b; Yeo et al., 2009; Aguado et al., 2003; Carmona et al., 2006).

In support to this KER, recent studies have demonstrated that injured hippocampal neurons can survive and be regenerated through the same mechanism (Shulga et al., 2013). Indeed, after mature nerve injury, KCC2 is down-regulated and the GABA responses switch to depolarization, in a way similar to the early developmental stages. The rescue and re-generation of these neurons requires the switch of GABA from depolarization to hyperpolarization, a process driven by BDNF and the subsequent KCC2 up-regulation in hippocampal neurons (Shulga et al., 2009) during brain development.

Empirical Evidence

It is widely accepted that BDNF expression is regulated by TH (Koibuchi et al., 1999; 2001; Chakraborty et al., 2012). Deregulation of BDNF signaling has been shown to decrease cortical GABA interneuron markers (Kelsom and Lu, 2013; Fiumelli et al., 2000; Arenas et al., 1996; Jones et al., 1994).

- Westerholz et al., 2013 In recent *in vitro* studies in rat T3-deficient cultures of cortical PV+ interneurons, it was shown that the number of synaptic boutons was reduced, an effect that was abolished after exogenous BDNF application. Additionally, inhibition of BDNF by K252a (a TrkB antagonist) in cultures containing T3 resulted also in decreased number of synaptic boutons, as in the T3-deprived cultures. These results suggest that BDNF signaling promotes the formation of synaptic boutons and that this function is mediated by TH (T3 and T4).

- Chen et al., 2016 BDNF-Val66Met knock-in mice (BDNF^{Met/Met}) are known for reduction in the activity-dependent BDNF secretion and elevated anxiety-like behaviors. This study showed that GABAergic innervation of pyramidal neurons of BDNF^{Met/Met} mice are reduced at distal dendrites in hippocampal CA1 and medial prefrontal cortex, compared to wild type mice.

- Kong et al., 2014 This study showed that chronic seizure rats 6 months after treatment with cyclothiazide (CTZ, a seizure inducer), underwent decrease of both GAD (from 75.2 ± 13.0 in CA1, 79.7 ± 9.7 in CA3, and 251.5 ± 4.3 in DG, respectively, to 3.0 ± 0.5 in CA1, 3.6 ± 0.9 in CA3, and 5.3 ± 1.8 in DG) and GAT-1 (from 60.7 ± 3.0 in CA1, 55.7 ± 9.1 in CA3, and 212.3 ± 11.3 in DG, respectively, to 20.7 ± 8.6 in CA1, 24.3 ± 3.4 in CA3, and 24.7 ± 13.3 in DG) across CA1, CA3, and dentate gyrus area of the hippocampus. Also, hippocampal decrease of both BDNF⁺ cells (from 70.7 ± 9.0 in CA1, 72.2 ± 3.7 in CA3, and 138.3 ± 15.9 in DG, respectively, to 4.1 ± 1.0 in CA1, 2.9 ± 0.1 in CA3, and 21.2 ± 16.2 in DG) and TrkB⁺ (BDNF receptor) cells (from 126.7 ± 7.2 in CA1, 275.7 ± 56.3 in CA3, and 399.2 ± 22.4 in DG, respectively, to 64.7 ± 16.2 in CA1, 158.3 ± 41.7 in CA3, and 250.3 ± 46.8 in DG) was observed.

- Aguado et al., 2003 BDNF overexpression in transgenic embryos raised the spontaneous activity of E18 hippocampal neurons, as shown by increased number of synapses (63% more synapses in the hippocampus of BDNF transgenic embryos than in controls), and increased spontaneous neuronal activity (2.3 times more active neurons than wild type embryos, and 36.3% greater rates of activation). Moreover, BDNF transgenic embryos had higher number of GABAergic interneuron synapses, as shown by higher GAD67 mRNA (by 3-fold) and K⁺/Cl⁻ KCC2 mRNA expression (by 4.3-fold) (responsible for the conversion of GABA responses from depolarizing to inhibitory), without altering the expression of GABA and glutamate ionotropic receptors. These data indicate that BDNF controls both GABAergic pre- and postsynaptic sites.

Uncertainties and Inconsistencies

The role of BDNF on differentiation and maturation of GABAergic interneurons is supported by the studies described in Weight of Evidence section. However, in a recent publication (Puskarjov et al., 2015) BDNF-/- mice were utilized to show that in the absence of BDNF the seizure-induced up regulation of KCC2 was eliminated, but interestingly no change in early (P5-6) or later (P13-14) postnatal KCC2 expression was observed compared to the wild type littermates, but neither the functionality of KCC2 protein was investigated, nor the ability of the neurons to extrude Cl⁻ in the absence of BDNF.

Additionally, other studies have shown that the up-regulation of KCC2 via the transcription factor Egr4 is also regulated by a different neurotrophic factor, neurturin (Ludwig et al., 2011b). These results reveal that the same transcriptional pathways, such as KCC2, can be activated by different neurotrophic factors and might lead to the same outcome under different conditions. This hypothesis should be further investigated, as it could explain the compensation mechanisms that are activated in the total absence of BDNF, and which might be different from those that are triggered by a decrease of BDNF levels.

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Relationship: 871: GABAergic interneurons, Decreased leads to Synaptogenesis, Decreased (<https://aopwiki.org/relationships/871>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	<i>Mus musculus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
Xenopus laevis	<i>Xenopus laevis</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8355)

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

Most of the available studies have been performed in rodent models and human cortical neurons, referenced in the "Biological plausibility" section.

The relationship between KCC2 function and GABA signalling has been also demonstrated in the retinotectal circuit of Xenopus (Akerman and Cline, 2006).

Key Event Relationship Description

Early in cortical development, the GABAergic interneurons have been found to contribute to key aspects of the brain development. A precise balance between excitatory and inhibitory synapses in cortical neurons is crucial for the formation and maturation of the neuronal connections and eventually the proper neural circuitry function. In the cerebral cortex, the young neurons first receive GABAergic depolarizing inputs before forming any synapses (Owens et al., 1999; Tyzio et al., 1999; Hennou et al., 2002), and thus the GABAergic system is believed to be the initial regulator of synaptogenesis. Indeed, initial depolarizing GABAergic transmission is required for the formation of the glutamatergic synapses and is therefore responsible for the regulation of the balance between excitation and inhibition in the developing cortex (Wang and Kriegstein, 2009; Owens et al., 1999; Tyzio et al., 1999; Hennou et al., 2002; Ben-Ari, 2006). Nascent GABAergic synapses contain both presynaptic and postsynaptic elements, and produce synaptic transmission (Ahmari and Smith, 2002). GABA_A receptors form clusters before presynaptic terminals emerge (Scotti and Reuter, 2001), and this clustering occurs in the absence of scaffolding proteins and GABA release (Scotti and Reuter, 2001; Christie et al., 2002). Also, during maturation, GABA_A receptors become selectively clustered across from terminals that release the neurotransmitter GABA (Craig et al., 1994; Swanwick et al., 2006).

Evidence Supporting this KER

Biological Plausibility

Early in the development of the neocortex, GABAergic interneurons play a role in the formation of spontaneous synchronized activity, which has a fundamental role in the activation of glutamatergic synapses, the synchronization of synaptogenesis and the establishment of long-range cortico-cortical connections (Voigt et al., 2001; 2005). Increasing evidence suggests that GABAergic signaling is the main regulator of this early neuronal activity, as it is established before the glutamatergic one in the neocortex (Wang and Kriegstein, 2009; Owens et al., 1999; Tyzio et al., 1999; Hennou et al., 2002; Ben-Ari, 2006). Despite the fact that GABA is the main inhibitory neurotransmitter in the adult CNS, it exerts depolarizing actions in the immature brain (Ben-Ari et al., 2006), caused by the low levels of Cl⁻ concentration in the post-synaptic cells (Rivera et al., 1999; Ehrlich et al., 1999). K-Cl co-transporter 2 (KCC2) is the main Cl⁻ efflux mechanism with a developmentally-regulated expression profile in the brain and it is therefore thought to be the regulator of GABA signalling during early neuronal development. The effects of KCC2 on the levels of [Cl⁻] in immature neurons and the subsequent effects on the shift of the GABA signaling has been extensively studied during the last decades:

- Existing data indicate that KCC2 expressed by GABA neurons is sufficient to shift from the depolarizing and excitatory period of GABA during cortical neuron development (Lee et al., 2005; Chudotvorova et al., 2005) and to effectively decrease the [Cl⁻] in immature rat neurons (Chudotvorova et al., 2005).
- Transcriptional repression of KCC2 in rat cortical neurons delayed the GABA switch corresponding to significant changes of [Cl⁻] in the same neurons (Yeo et al., 2009).

Several studies focused on the effects of GABA signaling on synaptogenesis and they all had convergent results leading to a strong biological plausibility of this KER.

- Too early shift of GABA-induced excitation-to-inhibition not only affects synaptic integration, but it also results in deficient circuitry development (Wang and Kriegstein, 2008). This has been demonstrated in rodents and mammals cortical neurons in culture.

Premature GABA switch has also morphological effects in cortical neurons, as it has been shown to drive in fewer and shorter dendrites with defective effects in synaptic formation (Cancedda et al., 2007).

- In the dentate gyrus of the adult hippocampus, newborn granule cells are tonically activated by ambient GABA before being sequentially innervated by GABA- and glutamate-mediated synaptic inputs. GABA initially exerts an excitatory action on newborn neurons owing to their high cytoplasmic Cl⁻ ion content (Ge et al., 2006).

- An early hyperpolarizing shift in Cl⁻ reversal potential, by premature expression of KCC2, has been shown to increase the ratio of inhibitory-to-excitatory inputs both in Xenopus tectal neurons and rat cortical neurons *in vitro* (Chudotvorova et al., 2005; Akerman and Cline, 2006).

The mechanistic details of this relationship are not entirely known, but the most possible mechanism entails a functional relationship between GABA and NMDA receptor activation (Wang and Kriegstein, 2008; Cserép et al., 2012). Cortical neurons begin to express functional NMDA receptors when they migrate to the cortical plate, but these initial glutamatergic synapses are "silent" because of the Mg²⁺ block of NMDA receptors at the resting membrane potential (LoTurco et al., 1991; Akerman and Cline, 2006). GABAergic depolarization can facilitate relief of this voltage-dependent Mg²⁺ block and allow Ca²⁺ entry to initiate intracellular signalling cascades (Leinekugel et al., 1997). This mechanism suggests that the initial depolarizing GABAergic transmission is required for the formation of the glutamatergic synapses and is therefore responsible for the regulation of the balance between excitation and inhibition in the developing cortex (Wang and Kriegstein, 2009).

Empirical Evidence

The correlation between the GABA function and synaptogenesis has been mainly studied through the developmental modifications of intracellular Cl⁻ gradient and the subsequent GABA switch. In all available cases, this is performed by disturbing KCC2 or NKCC1 expression with genetic or mechanical manipulations of the neuronal models.

The temporal concordance of GABA shift and synaptogenesis is extensively reviewed by Ben-Ari et al., 2007 and 2012. It is widely accepted that the first spontaneous synaptic activity in the cortex is driven by the GABA-mediated depolarization and it is necessary for the subsequent synapse formation in the brain. Furthermore, the absence of T3 in cultures of cortical GABAergic interneurons can delay the typical developmental KCC2 up-regulation and subsequently the GABA shift, with a profound decrease in the number of synapses (Westerholz et al., 2010; 2013).

- Westerholz et al., 2013 This study showed that in rat T3-deficient cultures of cortical GABAergic PV⁺ interneurons, the number of synaptic boutons (presynaptic terminals containing the presynaptic marker synaptophysin) was reduced, an effect that was abolished after exogenous BDNF application.

- **López-Espíndola et al., 2014:** Human brain sections from MCT8-deficient subjects (30th gestational week male fetus and an 11-year-old boy) were studied in comparison with relevant healthy control brain tissues. The MCT8-deficient fetal cerebral cortex showed 50% reduction of TH (i.e., T4, T3, and rT3), while T3 and T4 levels were normal in the liver. This TH deficiency in the brain produced an expected increase in type 2 deiodinase and decrease in type 3 deiodinase mRNA expression. Also, MCT8-deficient fetus showed a delay in cortical and cerebellar development and myelination, loss of parvalbumin (marker of GABAergic interneurons) expression, abnormal calbindin-D28k content, impaired axonal maturation (impaired synaptogenesis), and diminished biochemical differentiation of Purkinje cells. The 11-year-old boy displayed altered cerebellar structure, deficient myelination, deficient synaptophysin (presynaptic marker of synapse) and parvalbumin expression and abnormal calbindin-D28k expression.

Possible indirect KERs (not created due to limited evidence)

- **Fisher et al., (2013),** recently published a quantitative biologically-based dose-response model (BBDR) for iodine deficiency in the rat. In particular, HPT axis adaptations to dietary iodide intake in euthyroid (4.1-39 µg iodide/day) and iodide-deficient (0.31 and 1.2 µg iodide/day) conditions were evaluated. In rat pups that were iodide deficient during gestation and lactation, decreases in serum T4 levels were associated with declines in TH levels in the fetal brain. A 15% reduction in cortical T4 in the fetal brain was sufficient to induce permanent reductions in synaptic function in adults, confirming that even modest developmental TH disruption can cause synaptic dysfunctions also in hippocampal region (critical for learning and memory) of the adult brain (Gilbert et al., 2013). These data support indirect KER between decrease TH level in the brain and decreased synaptogenesis.

In regards to toxicological studies, bisphenol A (BPA), an environmental toxicant known to inhibit NIS-mediated iodide uptake (Wu Y et al., 2016), has also been found to delay and decrease both KCC2 expression and the developmental Cl⁻ shift (Yeo et al., 2013):

- **Yeo et al., 2013** In primary rat cortical neurons and primary human cortical neurons (obtained from human fetal brain tissue specimens), 100 nM BPA caused decrease of KCC2 mRNA expression ($\geq 25\%$ decrease in rat cells at 4-5 DIV, ~ 70% decrease in human cells at 10 DIV) and attenuated [Cl⁻] shift in migrating cortical inhibitory precursor neurons. These data support indirect KER between NIS inhibition and GABAergic interneuron alteration.

These findings also concur with studies in other brain areas, such as the auditory brainstem and the hippocampus (Friauf et al., 2008; Hadjab-Lallement et al., 2010), supporting correlation between KCC2 expression and GABA presence in the brain, and their implication in synaptogenesis.

Uncertainties and Inconsistencies

In vivo evidence for the role of GABA in synaptogenesis is controversial. Ji et al., 1999 have shown that in GAD65/67-deficient mice, in which the production of GABA was reduced to less than 5%, the development of brain morphology until birth was normal. These mice die at birth and therefore synaptogenesis and circuit development could not be controlled, however no morphological defects were detected in the neocortex, cerebellum and hippocampus of these animals by the time of their death. The authors of this study suggested that GABA may not be crucial for development. However, functional changes were not assessed in this study. One hypothesis is that glutamate, glycine and taurine could compensate for the lack of GABA (LoTurco et al., 1995; Flint et al., 1998).

In KCC2 knock out mice, apart from lung atelectasis, no other obvious histological changes in the brain were observed in neonatal mice (Hubner et al., 2001). Moreover, these mice died at birth, before the GABA switch takes place, and neuronal electrical activity or synaptogenesis were not evaluated.

Additionally, after premature expression of KCC2 transporter an increase of the excitatory synapses was observed, but the glutamatergic synapses were not affected (Chudotvorova et al., 2005), as in the case of NKCC1 knock out mice (Wang and Kriegstein, 2008). These contradictory results reveal the complexity of the developmental brain and suggest that many different mechanisms are involved in the regulation of the temporal profile of the two main neuronal co-transporters, namely the NKCC1 and KCC2. However, in all cases the importance of Cl⁻ homeostasis in the developmental cortex and its correlation with the proper synapse formation is demonstrated.

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Relationship: 358: Synaptogenesis, Decreased leads to Neuronal network function, Decreased (<https://aopwiki.org/relationships/358>)

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 induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	adjacent	Low	
Inhibition of Na ⁺ /I ⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	<i>Homo sapiens</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	<i>Rattus norvegicus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	<i>Mus musculus</i>	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
<i>Caenorhabditis elegans</i>	<i>Caenorhabditis elegans</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=6239)
<i>Drosophila melanogaster</i>	<i>Drosophila melanogaster</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7227)

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

The main proof of evidence comes from *in vivo* studies in rodents. However, Colón-Ramos (2009) has recently reviewed the early developmental events that take place during the process of synaptogenesis in invertebrates, pointing out the importance of this process in neural network formation and function. The experimental findings reviewed in this paper derive from knowledge acquired in the field of neuroscience using *C. elegans* and *Drosophila*; at the same time, emerging findings derived from vertebrates are also discussed (Colón-Ramos, 2009).

Key Event Relationship Description

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 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 dendrite arbor 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. Since synaptogenesis follows the early neurodevelopmental processes such as neuronal and glial cells proliferation, migration, alterations in dendrite arborisation etc., therefore, it encompasses, possible changes in these early stages of brain development that could also be triggered under hypothyroidism, leading to defective synaptogenesis and resulting in abnormal function of neuronal network function. These anatomical alterations are responsible for many structural anomalies reported in various regions of the brain following severe developmental hypothyroidism. Although the primary evidence of synaptic transmission impairments in hypothyroid models have come from studying the hippocampus, it is assumed that the role thyroid hormones play in these processes is likely similar across different brain regions. Altered hippocampal structure induced by decreased TH levels impacts neurogenesis in the developing hippocampus or cortex, contributing to deficits in synaptic function.

Evidence Supporting this KER

The weight of evidence supporting the relationship between decreased synaptogenesis induced by TH insufficiency and altered neuronal network and synaptic function is moderate. Functional change as exemplified by alterations in synaptic transmission may be more easily detected than structural abnormalities. The exact alignment between the neuroanatomical effects (such as decreased synaptogenesis and alteration of GABAergic interneurons) that have been associated with developmental hypothyroidism (e.g., elicited by exposing rat dams to TPO inhibitors) and the neurophysiological impairments has not been entirely elucidated.

Biological Plausibility

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).

The biological plausibility of the known effects of TH insufficiency on brain structure having an impact on synaptic function and plasticity in brain is strong. Reductions in myelination of axons, cell number, dendrite arborization, and synaptogenesis have been described in models of severe hormone deprivation, as comprehensively summarized by Thompson and Potter, 2000. Because synaptic transmission relies on the integrity of synaptic contacts and the electrical and chemical transmission between pre- and post-synaptic neurons, it is well accepted that interference with process of synapse formation (morphological unit of neuronal network) will very much impact the neural network function.

Empirical Evidence

Most of the information on developmental hypothyroidism and altered synaptic function has been derived from studies of the hippocampus. It is presumed that structural changes of synaptic connectivity at certain level may lead to functional deficits in synaptic transmission and plasticity impairments (Vara et al., 2002, Sui and Gilbert, 2003, Gilbert, 2004, Dong et al., 2005, Sui et al., 2005), but the precise structural aberration is not known. Within the hippocampus, area CA1 has been investigated primarily with *in vitro* techniques, using slices of hippocampus from animals exposed to TPO inhibitors (MMI or

PTU) and measuring synaptic function across CA1-pyramidal cell synapses (Vara et al., 2002, Sui and Gilbert, 2003, Gilbert, 2004, Taylor et al., 2008). Pyramidal neurons of hypothyroid animals have fewer synapses and an impoverished dendritic arbor (Rami et al., 1986a, Madeira et al., 1992), with reductions ranging between 14.2 and 22.5% as observed in 30 and 180-day-old hypothyroid rats (as described by Madeira et al., 1992).

The other major region in hippocampus investigated in hypothyroid models is the perforant path-dentate gyrus synapse (Gilbert, 2011). Granule cells are the principal cell type of the dentate gyrus region of the hippocampal formation and receive input from cortical neurons in the entorhinal cortex. TPO inhibitors like PTU and MMI decrease the volume of the granule cell layer, the density of cells within the layer, and estimates of total granule cell number (Madeira et al., 1991). Migration of granule cells from the proliferative zone to the granule cell layer is retarded by thyroid deficiency as is dendritic arborization and synaptogenesis assessed by immunohistochemistry for the synaptic protein, synaptophysin (Rami et al., 1986b, Rami and Rabie, 1990, Dong et al., 2005). Impairments in synaptic function from both rodent and human studies are summarized below.

Excitatory and inhibitory synaptic transmission is reduced in CA region of hippocampus in animals with TH insufficiencies in early life (Vara et al., 2002, Sui and Gilbert, 2003, Gilbert, 2004, Dong et al., 2005, Sui et al., 2005). Similarly, excitatory and inhibitory synaptic transmission is reduced in the CA1 and dentate gyrus regions of the hippocampus (Gilbert and Paczkowski, 2003, Gilbert and Sui, 2006, Gilbert, 2011, Gilbert et al., 2013) under decreased TH levels. Parvalbumin (PV) is a calcium-binding protein expressed exclusively in GABA inhibitory neurons of the hippocampus. Impairments in inhibitory synaptic transmission are associated with reductions in the number of PV⁺ cells (Gilbert et al., 2007).

- Vara et al., 2002 This study assessed effects of TH on short-term synaptic plasticity (associated with short-term memory) in control vs hypothyroid rats. Specifically, dams were treated with 0.02% methylmercaptoimidazole (MMI, TPO inhibitor) continually from GD9. A group of pups were also treated with T3 (i.p. daily injections of T3 (20 µg /100 g body weight) starting 72 h before killing. Data showed that hypothyroid rats presented increase in the Ca(2+)-dependent neurotransmitter release, indicative of altered neuronal network function (i.e., decreased paired-pulse facilitation), and these alterations were reverted by T3 administration. These synaptic changes were determined by an increase of synapsin I and synaptotagmin I levels in the hypothyroid rats, suggesting that TH modulate neurotransmitter release. These results are in contrast with those of Di Liegro et al. (1995), who showed that in primary cultures T₃ induces the expression of synapsin I. This difference could be the result of the multiple differences between the two models: different brain regions (hippocampus vs. cortex), the age of the neurons (neonatal vs. embryonic), the preparations (slice vs. culture).

- Sui and Gilbert, 2003 Here developing rats were exposed in utero and postnatally to 0, 3, or 10 ppm propylthiouracil (PTU, TPO inhibitor), administered in the drinking water of dams from GD6 until PND30. Excitatory postsynaptic potentials and population spikes (indicative of neuronal network function) were recorded in area CA1 of hippocampal slices from offspring between PND21 and PND30. PTU caused at PND30 a decrease of maternal total T₄ (43.9% with 3-ppm, and 65.0% with 10-ppm) compared to controls, with no change in total T₃. Maternal TSH was increased above control levels in a dose-dependent manner. In pups, total T₄ was depressed (by 75%) in both treated groups relative to the controls pups, and total T₃ was depressed (35.8% in the 3-ppm group and 66.5% in the 10-ppm group) relative to the controls. TH insufficiency was dose-dependently associated with a reduction of paired-pulse facilitation and long-term potentiation of the excitatory postsynaptic potential and elimination of paired-pulse depression of the population spike. Excitatory synaptic transmission was increased by developmental exposure to PTU. This suggests that TH insufficiency compromises synaptic communication in area CA1 of developing rat hippocampus (involved in learning and memory).

- Gilbert, 2004 Here developing rats were transiently exposed to PTU (0 or 15 ppm), through the drinking water of pregnant dams beginning on GD18 until PND21. This regimen markedly reduced circulating levels of TH in pups (T₃: ~50% lower than control at PND21; T₄: T₄ ~40% below control levels). Analysis of field potentials in area CA1 of hippocampal slices derived from adult male offspring exposed to PTU showed a reduction of somatic population spike amplitudes, with no differences in excitatory postsynaptic potentials (EPSP). Short-term plasticity of the EPSP (as indexed by paired pulse facilitation) was markedly decreased by PTU exposure. These data confirm associations between decrease of synaptic function in the hippocampus and decrease of neuronal network function as a consequence of TH insufficiency.

- Dong et al., 2005 Through gestation and lactation, iodine-deficient or hypothyroid dam rats were administered with either iodine-deficient diet or MMI (TPO inhibitor) added to drinking water. Exposure was terminated on PND30. Both treated groups showed lower concentrations of serum FT3 (~60% decrease on PND30) and FT4 (~80% decrease on PND30), smaller population spike amplitude (~50% decrease) and field-excitatory postsynaptic potential (f-EPSP, 50-60% decrease) slope induced by high-frequency stimulation (HFS). Also, TH insufficiency decreased the levels of c-fos (by ~60% at PND30 in MMI group) and c-jun proteins (by ~20% at PND30 in MMI group) in the hippocampus. C-fos and c-jun expression is regulated by synaptic activity, both play an important role in the neuroplastic mechanisms (synaptogenesis) critical to memory consolidation, and play an essential role in neuronal differentiation (Herdegen et al., 1997).

KEs proceeding the AO (learning and memory deficits), such as "Decreased synaptogenesis" (KEup) and "Decreased Neural Network Function" (KEdown) are 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> (<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 (Decreased synaptogenesis leads to decreased neuronal network function in developing brain) described in the present AOP.

- Otto and Reiter, 1984: At low Pb²⁺ levels (less than 30 µg/dl), slow surface-positive cortical potentials have been observed in children under five years old, which may reflect axodendritic inhibitory processes, whilst negative slow potentials are observed in children over five years. This is due to the fact that during maturation of the cortex, the locus of inhibitory activity shifts deeper to axosomatic connections. However, age-related polarity reversal has been observed in children with higher Pb²⁺ levels.

- Kumar and Desiraju, 1992: In experiments carried out in Wistar rats that have been fed with lead acetate (400 µg/g body weight/day) from PND 2 until PND 60, EEG findings show statistically significant reduction in the delta, theta, alpha and beta band of EEG spectral power in motor cortex and hippocampus with the exception of the delta and beta bands power of motor cortex in wakeful state.

- McCarron and Eccles, 1983: Male Sprague-Dawley rats have been exposed to Pb²⁺ from parturition to weaning though their dams' milk (dams received drinking water containing 1.0, 2.5, or 5.0 mg/ml lead acetate). Starting from 15 weeks of age, the characteristics of the electrically elicited hippocampal after discharge (AD) and its alteration by phenytoin (PHT) showed significant increase in primary AD duration only in the animals exposed to the higher dose of Pb²⁺, whereas all groups responded to PHT with increases in primary AD duration.

The exact mechanism by which a change in cell number, reduced dendritic arborization and synaptogenesis may lead to decreased neuronal network function has not been fully elucidated. Dose-dependent reductions in synaptic function in hippocampus have been demonstrated in models of moderate degrees of TH reduction, but studies of the anatomical integrity of the specific cell populations examined electrophysiologically have largely been evaluated in models of severe hypothyroidism and often in brain regions distinct from the hippocampus.

Uncertainties and Inconsistencies

The exact mechanism by which a change in cell number, reduced dendritic arborization and synaptogenesis may lead to decreased neuronal network function has not been fully elucidated. Dose-dependent reductions in synaptic function in hippocampus have been demonstrated in models of moderate degrees of TH reduction, but studies of the anatomical integrity of the specific cell populations examined electrophysiologically have largely been evaluated in models of severe hypothyroidism and often in brain regions distinct from the hippocampus.

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Relationship: 359: Neuronal network function, Decreased leads to Impairment, Learning and memory (<https://aopwiki.org/relationships/359>)

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 induces impairment of learning and memory abilities (https://aopwiki.org/aops/13)	non-adjacent	Low	
Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony loss/failure 2 (https://aopwiki.org/aops/90)	adjacent		
Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1 (https://aopwiki.org/aops/78)	adjacent		
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	adjacent	High	Low
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)	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

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 Dorsey, 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 (not always and not always high frequency stimulation leads to LTP), 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 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> (<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 Pb2+ through gestation and lactation have shown more severe impairment of memory than male rats with similar Pb2+ exposures.

- **De Souza Lisboa et al., 2005** This study reported that exposure to Pb2+ 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 Pb2+-exposed rats (250, 750 and 1500 ppm Pb2+ acetate in food) during gestation and through weaning and demonstrated that these outcomes are very much influenced by sex and rearing environment. In females, Pb2+ exposure lessened some of the benefits of enriched environment on learning, whereas, in males, enrichment does help to overcome detrimental effects of Pb2+ on learning. Regarding reference memory, environmental enrichment has not been beneficial in females when exposure to Pb2+ occurs, in contrast to males.

- Jaako-Movits et al., 2005 Wistar rat pups were exposed to 0.2% Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ exposure (Cory-Slechta, 1995; Cohn and Cory-Slechta, 1993 and 1994).

- Xiao et al., 2014 Rat pups from parents exposed to 2 mM PbCl₂ three weeks before mating until their weaning (pre-weaning Pb²⁺) and weaned pups exposed to 2 mM PbCl₂ for nine weeks (post-weaning Pb²⁺) were assessed for their spatial learning and memory by MWM on PND 85-90. The study revealed that both rat pups in pre-weaning Pb²⁺ and post-weaning Pb²⁺ groups performed significantly worse than those in the control group. The number of synapses in pre-weaning Pb²⁺ group increased significantly, but it was still less than that of control group. The number of synapses in post-weaning Pb²⁺ group was also less than that of control group, although the number of synapses had no differences between post-weaning Pb²⁺ and control groups before MWM. In both pre-weaning Pb²⁺ and post-weaning Pb²⁺ 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> (<https://aopwiki.org/aops/13>)). 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 microg/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 injected 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 weight). 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 the 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 day 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 visual 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 the how these two KEs are connected.

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.

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. However, Pb exposure is not always associated with learning and memory impairment in children. In this regard, Koller's review has commented that in some occasions, low-level Pb dose and cognitive deficits of the subjects are negatively correlated, and this may be due to the high influence of social and parenting factors in cognitive ability, like learning and memory (Koller et al., 2004).

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 field measurement), 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.

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List of Non Adjacent Key Event Relationships

Relationship: 1503: Inhibition, Na⁺/I⁻ symporter (NIS) leads to Impairment, Learning and memory (<https://aopwiki.org/relationships/1503>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	non-adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

As described in the *Empirical Support* section, the association between NIS inhibition and learning and memory impairment has been studied only in rodent models and in humans.

Key Event Relationship Description

NIS is a membrane protein responsible for iodide transport into the follicular cells of the thyroid, which is the first and most critical step leading to T4 biosynthesis (Dohan et al., 2000). TH synthesis is dramatically suppressed in case of NIS dysfunction or inhibition (Spitzweg and Morris, 2010; Jones et al., 1996; Tonacchera et al., 2004; De Groot et al., 2006), resulting in the decreased TH levels in the serum and consequently in the brain. Hypothyroid brain development results in severe functional impairments including ataxia, spasticity, severe mental retardation, including impairment of learning and memory.

NIS inhibition occurring as a consequence of exposure to certain pollutants has been associated with learning and memory deficits in rodents and humans (Wang et al., 2016; Jang et al., 2012; Taylor et al., 2014; Chen et al., 2014; Roze et al., 2009; van Wijk et al., 2008; Wu Y et al., 2016).

Evidence Supporting this KER

The weight of evidence supporting an indirect linkage between the MIE, NIS inhibition, and the adverse outcome *Impairment of learning and Memory* is moderate.

Biological Plausibility

NIS inhibition occurring as a consequence of exposure to certain pollutants has been associated with learning and memory deficits in rodents and humans (Wang et al., 2016; Jang et al., 2012; Taylor et al., 2014; Chen et al., 2014; Roze et al., 2009; van Wijk et al., 2008).

During pre- and perinatal development, disruption of TH signaling leads to a multitude of neurological deficits. Multiple studies have shown that TH deprivation leads to defects in learning processes (for a comprehensive review, see Raymaekers and Darras, 2017). Congenital hypothyroidism has been shown to cause selective visuospatial malfunctions, a lower IQ even in young adults (Oerbeck et al., 2003; Simic et al., 2013; Wheeler et al., 2012; Willoughby et al., 2014). On the other hand, adult-onset hyperthyroidism has been associated with a decrease in signal activity between the hippocampus and other cortical regions (Zhang et al., 2014), hyperactivity, attention deficits and changes in anxiety state (Raymaekers and Darras, 2017), which could impact learning potential.

Empirical Evidence

Some epidemiological and *in vivo* studies have indicated associations between NIS inhibition (e.g., as a consequence of exposure to perchlorate or other pollutants, such as BPA and BDE-47, NIS inhibitors) (Wu Y et al., 2016) and decreased cognition.

BPA exposure has been also associated with hypothyroidism (i.e., decreased of free T3 and free T4, increase of TSH plasma levels, perturbation of thyroid gland morphological structure and thyroid cell function) in humans (i.e., inverse relationships between urinary BPA and total T4 and TSH) (Meeker and Ferguson, 2011), in young rats breast-fed from mothers treated with BPA (Mahmoudi et al. 2018), and in pregnant ewes and their newborn lambs (i.e., decrease of total T4 in BPA-treated pregnant ewes and in the cord and the jugular blood of their newborns (30% decrease), and of plasma free T4 levels in the jugular blood of the newborns) (Viguer et al. 2013).

PBDEs and their hydroxylated metabolites (OH-PBDEs) can bind to the serum-binding proteins transthyretin and thyroxine-binding globulin, can affect deiodinases (DI 1, 2 and 3) activity, and alter TH metabolism and excretion, leading to hypothyroidism in experimental animals (Butt et al. 2011; Marchesini et al. 2008; Meerts et al. 2000; Szabo et al. 2009; Zhou et al. 2002). Human studies also observed PBDE-associated TH disruption during pregnancy (Chevrier et al. 2010; Herbstman et al. 2008; Lin et al. 2011; Stapleton et al. 2011; Zota et al. 2011). Therefore, thyroid disruption may be a critical underlying mechanism related to the developmental neurotoxicity of PBDEs and their metabolites (Dingemans et al. 2011; Costa et al. 2008; Chen et al. 2014).

- Wang et al., 2016: in this *in vivo* study, pregnant Sprague-Dawley female rats were orally treated with either vehicle or BPA (0.05, 0.5, 5 or 50 mg/kg BW/day) during days 9-20 of gestation. Male offspring were tested on PND 21 with the object recognition task. BPA-exposed male offspring underwent memory and cognitive impairments: they not only spent more time (~ 43% more, at 1.5 hr after training) in exploring the familiar object at the highest dose than the control, but also displayed a significant decrease in the object recognition index (at 50 mg/kg BW/day, ~ 54% lower short term memory measured

1.5 hr after training).

- **Jang et al., 2012:** In this *in vivo* 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. 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. These results suggest that BPA exposure (NIS inhibition) in pregnant mothers could decrease hippocampal neurogenesis and cognitive function in future generations.

- **Taylor et al., 2014:** In this historical cohort study of 21,846 women in Cardiff, United Kingdom, and Turin, Italy, who were pregnant from 2002 to 2006, levels of urinary perchlorate (a NIS inhibitor) in the highest 10% were associated with a higher risk for having children with IQ scores in the lowest decile at age three, as described in 487 mother-child pairs in mothers who were hypothyroid/hypothyroxinemic during pregnancy.

- **Chen et al., 2014:** In this prospective birth cohort, maternal serum concentrations of BDE-47 and other PBDE congeners were measured in 309 women at 16 weeks of gestation, and associated with neurodevelopment in children. Importantly, BDE-47 and other chemicals, such as triclosan, triclocarban and BPA, have been reported to disturb TH homeostasis by inhibiting NIS-mediated iodide uptake and altering the expression of genes involved in TH synthesis in rat thyroid follicular FRTL-5 cells (Wu Y et al., 2016). A 10-fold increase in prenatal BDE-47 exposure was associated with a 4.5-point decrease in Full-Scale IQ and a 3.3-point increase in the hyperactivity score at age 5 years in children.

- **Roze et al., 2009:** Similarly, this epidemiological study assessed the level of several compounds, including BDE-47 (i.e., 2,2'-bis-(4 chlorophenyl)-1,1'-dichloroethene, pentachlorophenol (PCP), PCB-153, 4OH-CB-107, 4OH-CB-146, 4OH-CB-187, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, and hexabromocyclododecane), in 62 mothers during the 35th week of pregnancy, and possible associations with the neuropsychological level in their children at 5-6 years of age. THs were determined in umbilical cord blood. Brominated flame retardants correlated with worse fine manipulative abilities, worse attention, better coordination, better visual perception, and better behavior. Chlorinated OHCs correlated with less choreiform dyskinesia. Hydroxylated polychlorinated biphenyls correlated with worse fine manipulative abilities, better attention, and better visual perception. The wood protective agent (PCP) correlated with worse coordination, less sensory integrity, worse attention, and worse visuomotor integration.

- **van Wijk et al., 2008:** This *in vivo* study assessed the behavioural effects of perinatal and chronic hypothyroidism during development in both male and female offspring of hypothyroid rats. To induce hypothyroidism, dams and offspring were fed an iodide-poor diet and drinking water with 0.75% sodium perchlorate (NIS inhibitor). Treatment was started in dams 2 weeks prior to mating, and in pups either until the day of killing (i.e., chronic hypothyroidism) or only until weaning (i.e., perinatal hypothyroidism) to test for reversibility of the effects observed. Early neuromotor competence, as assessed in the grip test and balance beam test, was impaired by both chronic and perinatal hypothyroidism. The open field test, assessing locomotor activity, revealed hyperactive locomotor behavioural patterns in chronic hypothyroid animals only. The Morris water maze test, used to assess cognitive performance, showed that chronic hypothyroidism affected spatial memory in a negative manner. Perinatal hypothyroidism was found to impair spatial memory in female rats only. In general, the effects of chronic hypothyroidism on development were more pronounced than the effects of perinatal hypothyroidism. This suggests that the early effects of hypothyroidism on functional alterations of the developing brain may be partly reversible.

- **Kosugi et al. 1998; Ferrandino et al. 2017:** Three Japanese children inherited two NIS mutations (V59E and T354P) from their healthy mother and father, respectively. V59E NIS was reported to exhibit as much as 30% of the activity of wild-type NIS (Fujiwara et al. 2000). The T354P and V59E NIS mutant proteins, when expressed in COS7 cells, were both trafficked to the cell surface, but totally inactive. The three siblings displayed different degrees of mental retardation, including heavy learning and memory deficits. The oldest one was nursed for longer than the second oldest, and evinced a less severe cognitive deficit. The youngest was not nursed, and displayed a more severe cognitive deficit than either of her siblings. It was discovered that the mother was addicted to laminaria, an alga extremely rich in I⁻ (Ferrandino et al. 2017). These studies will be also cited in support of Essentiality for KE (MIE).

- **Babu et al. 2011:** in this *in vivo* study 50-day-old female rats weighing 120–150 g were switched to a low iodine diet (LID) and given 1% KCIO4 (NIS inhibitor) in drinking water for 10 days. Animals were then separated into an iodine sufficient group (or euthyroid) and a low iodine diet (or hypothyroxinemic) group (0.005% KCIO4) and kept on above diet regimen for 3 months. Based on the hormonal estimations and urinary iodine, female rats were further divided into euthyroid and hypothyroxinemic and were mated with normal males. In a separate group of age-matched female rats, hypothyroidism was induced in rats by giving MMI (0.025% wt/vol) in drinking water to the pregnant rats from gestational day 8 and continued thereafter until sacrifice of pups born to these dams (hypothyroid group).

Data showed a significant reduction in total serum T4 and T3 levels of rat pups administered with MMI compared to euthyroid controls (3-fold decrease of T3 vs ctr and 7-fold decrease of T4 at P16). Hypothyroxinemic pups (on low iodine diet and KCIO4) showed a reduction in serum T4 (~ 70% decrease of T4 vs ctr) but not in T3, which was increased compared to euthyroid levels at P16 (~ 40% increase of T3 vs ctr). Even in the presence of elevated circulating T3 levels, hypothyroxinemic pups showed significantly impairment of TH responsiveness in developing rat neocortex.

Both hypothyroid (MMI) and hypothyroxinemic (KCIO4) pups demonstrated a significant increase in D2 levels compared to controls (~ 11 fold in hypothyroidism, and ~ 4 fold in hypothyroxinemia). The expression of D3 mRNA was also decreased significantly (by ~ 3.3 fold in hypothyroidism and ~ 3 fold in hypothyroxinemic group compared to controls), whilst MCT8 and TH nuclear receptors α 1 and β 1 expression did not change. Additionally, myelin basic protein (MBP) protein levels and gene were decreased in both groups (for MBP gene: by ~ 60% and ~ 70% respectively in hypothyroidism and hypothyroxinemic groups vs Ctr). Moreover, increased number of apoptotic neurons was found evenly distributed in all the layers of the neocortex under both hypothyroxinemic and hypothyroid conditions. As stated in this study, altogether these data suggest that hypothyroxinemia induced by low iodine diet and KCIO4 may lead to learning and memory impairment in this model. However, memory or cognitive tests were not assessed in this study.

- **Buras et al. 2014:** in this *in vivo* study 9-10 week old mice were administered with drinking water **containing 0.05% MMI and 1% KCIO4** for 4 weeks to render them hypothyroid. After 4 weeks, the hypothyroid group was further divided into 3 groups: the hypothyroid (0.05% MMI + 1% KCLO4), T3 (0.05% MMI + 1% KCL04 + T3 (0.5 μ g/ml) in drinking water) and T4 (0.05% MMI + 1% KCL04 + T4 (5 μ g/ml) in drinking water) groups for weeks 5 and 6. T3 serum levels were decreased by ~ 40% in hypothyroid group vs Ctr, and T4 was totally depleted in hypothyroid group vs Ctr. Several tests were performed to evaluate fear-anxiety behaviour. In the elevated plus maze, the hypothyroid mice showed significantly lower distance and time in the open arms than the T3-treated group (~ 50% for both parameters) than the euthyroid controls. The hypothyroid group also showed greater distance and time in the closed arms (~ 10% and 20% more than Ctr respectively for distance and time scores) than the T3-treated group. Administration of T3 and T4 rescued these effects. Moreover, hypothyroid mice froze more than Ctr (~ 35% more) and T3 and T4 treatments reversed this effect.

- **Navarro et al. 2015:** in this *in vivo* study 0.02% MMI and 1% KCIO4 were added to the drinking water in rats starting at embryonic day 10 (E10, developmental hypothyroidism) and E21 (early postnatal hypothyroidism) until day of sacrifice at PND 50. Behavior was studied using the acoustic prepulse inhibition (somatosensory attention) and the elevated plus-maze (anxiety-like assessment) tests. Total plasmatic T4 levels of both E10 (1.86 ng/ml) and E21 (1.08 ng/ml) pups were significantly lower than those of Ctr (36.29 ng/ml) pups. Total plasmatic T3 levels of E10 (0.10 ng/ml) and E21 (0.10 ng/ml) were significantly lower than in Ctr (0.45 ng/ml) pups. E10 and E21 treated pups showed abnormal laminar organization of the hippocampus, critical brain structure for learning and memory processes. The distribution, density and size of VGlut1 and VGAT boutons in the hippocampus and somatosensory cortex was abnormal in hypothyroid pups (in both groups) and these changes correlated with behavioral changes: prepulse inhibition of the startle response amplitude was reduced (23.3% in E10, 43.0% in E21 and 79.0% in Ctr pups), indicating severe pre-attention deficit in treated pups, while the percentage of time spent in open arms increased (57.0% time spent in open arms in E21 and 81.1% in E10 pups, vs 17.1% Ctr pups, indicative of increased anxiety).

- **Vasilopoulou et al. 2016:** this *in vivo* study investigated the effects of adult onset hypothyroidism (induced by administration of 1% w/v KCIO4 in their drinking water for 8 weeks in adult male Balb/cJ mice) on acetylcholinesterase (AChE) activity and on related behavioral parameters. They found that adult onset hypothyroidism (TH levels were not measured in this study) caused decrease of memory and increased fear/anxiety (i.e., 51% decrease of time spent in open arms / [times spent in open + closed arms], 47% decrease of the number of entries into the open arms of the apparatus, and 42% decrease in the total number of arm entries), and activity of both isoforms of AChE was reduced in all examined brain regions.

Uncertainties and Inconsistencies

Single NIS mutations, causing decreased thyroidal iodide uptake, may not necessarily lead to cognitive disorders. In this regard, Nicola and coworkers (Nicola et al., 2015) recently identified a new NIS mutation (V270E) in a patient (full-term girl born to healthy, non-consanguineous Jamaican parents), who resulted to be heterozygous for this NIS mutation (R124H/V270E). The presence of the mutation V270E markedly reduces iodide uptake (5.4% 24 hours after the oral administration of 100 μ Ci ¹²³I – (normal range, 10–40%)) via a pronounced (but not total) impairment of the protein's plasma membrane targeting. However, the retaining of a minimal iodide uptake was enough to enable sufficient TH biosynthesis and prevent cognitive impairment.

It should be noted that van Wijk et al. 2008 study was performed with only one dose group exposed to perchlorate during development, and the behavioural assessments were performed using a limited group size of 5-8, possibly reducing the reliability of this study. In general, chronic hypothyroidism effects on development were more pronounced than the effects of perinatal hypothyroidism, suggesting that functional alterations occurring as a consequence of hypothyroidism may be partly reversible depending on developmental stage of the deficiency.

Opposite, other *in vivo* studies do not support associations between perinatal perchlorate exposure and neurobehavioural effects. For example, York et al. (2004) could not observe meaningful behavioral effects in rat offspring exposed as high as 10.0 mg/kg/day, as evaluated by passive avoidance, swimming water maze, motor activity, and auditory startle. In their re-evaluation of the data (York et al. 2005), authors concluded that rat pups exposed to perchlorate both during pregnancy and after 10 days of lactation, despite showing alterations of neurohistopathological features, did not show altered development of gross motor movements. Moreover, Gilbert and Sui (2008) found that adult male offspring born from rat dams exposed to 0, 30, 300, or 1,000 ppm perchlorate in drinking water from gestational day 6 until weaning, underwent reduction of T3 (10–14% reduction) and T4 (~ 9–20% reduction) reduction on postnatal day 21 (at the highest perchlorate dose), significant reductions in baseline synaptic transmission (~ 20% increase in excitatory postsynaptic potential slope amplitude), but without changes of motor activity, spatial learning, or fear conditioning.

Taylor et al. 2004 (CATS study) identified 1050 pregnant women with hypothyroidism or hypothyroxinemia; half were in the immediate T4 treatment group, and half were in the group tested and treated after pregnancy. 487 (46.4%) mother-child pairs completed psychological testing and urinary iodine and perchlorate measurements. Therefore, the 487 women-child pairs represent approximately two-thirds of those reported in the study of T4 treatment effects on cognitive outcome. Taking this into account, the absence of a direct effect of perchlorate on maternal thyroid function (Pearce et al. 2010), suggests that developmental effects of perchlorate may not necessarily be linked to maternal thyroid hormone levels, as commented in (Brent, 2014).

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AOP54

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Relationship: 1506: TH synthesis, Decreased leads to Impairment, Learning and memory (<https://aopwiki.org/relationships/1506>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Deficiencies in learning and memory following developmental hypothyroidism (TH synthesis inhibition) have been documented mainly in rodents and humans.

Key Event Relationship Description

It is widely accepted that the thyroid hormones (TH) play a prominent role in the development and function of the CNS, including hippocampus and neocortex, two critical brain structure closely linked to the cognitive function (Gilbert et al., 2012). Brain concentrations of T4 are dependent on transfer of T4 from serum, through the vascular endothelia, into astrocytes. In astrocytes, T4 is converted to T3 by deiodinase and subsequently transferred to neurons cellular membrane transporters. In the brain T3 controls transcription and translation of genes responsible for normal hippocampal structural and functional development. Normal hippocampal structure and physiology are critical for the development of cognitive function. Thus, there is an indisputable indirect link between TH synthesis, controlling the levels of T4 in serum, and cognitive function, including learning and memory processes.

Evidence Supporting this KER

The weight of evidence supporting the relationship between decreased TH synthesis and learning and memory impairments (occurring as a consequence of altered neuronal network and synaptic function) is strong (Vara et al., 2002; Sui and Gilbert, 2003, 2004, 2011; Dong et al., 2005; Sui et al., 2005). This is consistent with the well understood and documented relationship between TH synthesis that is responsible for TH concentrations in serum, and consequently in brain. TH controls brain development and function, including learning and memory processes, in humans and animals.

Biological Plausibility

The importance of thyroid hormones (TH) in brain development has been recognised and investigated for many decades (Bernal, 2011; Williams 2008). Several human studies have shown that low levels of circulating maternal TH (as a consequence of a decrease of TH synthesis) can lead to neurophysiological deficits in the offspring, including learning and memory deficits, or even cretinism in most severe cases (Zoeller and Rovet, 2004; Henrichs et al., 2010).

Empirical Evidence

A number of studies have consistently reported alterations in synaptic transmission resulting from developmental TH disruption, and leading to decreased cognition. Developmental hypothyroidism reduces the functional integrity in brain regions critical for learning and memory. For example, pyramidal neurons of hypothyroid animals have fewer synapses and an impoverished dendritic arbor (Rami et al., 1986a; Madeira et al., 1992) that would lead to cognitive impairments.

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 deficits persist into adulthood long after recovery to euthyroid status.

Hypothyroidism, induced by different approaches, including inhibition of TH synthesis, 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). Impairments in synaptic function and plasticity are observed coincident with deficits in learning tasks that require the hippocampus.

Several *in vivo* studies have reported associations between decrease of TH synthesis (induced by TPO inhibitors) and learning and memory impairments:

- **Davenport and Dorsey, 1972; Tamasy et al., 1986:** In these *in vivo* studies, deficits in passive avoidance learning have been reported, but these early observations are often limited to animals suffering fairly severe hormonal deprivation.

- **Akaike, 1991:** in this *in vivo* study, temporary hypothyroidism was induced in neonatal rats by 0.02% PTU administration to lactating dams during days 0-19 after delivery. The radial arm maze test started at 13 weeks revealed that the PTU animals required more trials until they showed the first well-performed trial (with 3-fold more errors on day 3). The total number of choices was also larger, with less correct choices. These results suggest an involvement of temporary neonatal hypothyroidism in learning and memory impairment.

- **Axelstad et al., 2008:** in this *in vivo* study, radial arm maze deficits were recorded in adult offspring of rat dams treated with high doses of the TPO inhibitor, propylthiouracil (PTU), throughout gestation and lactation. Data showed a ~66% increase in the total number of errors made in the radial arm maze during 3 weeks of testing, in adult male rats perinatally exposed to 1.6 mg/kg/day of PTU from GD7 to PND17.

- Shafiee et al., 2016: This *in vivo* study investigated the effects of PTU (TPO inhibitor, 100 mg/L) in pregnant rats to evaluate the effects elicited by maternal hypothyroidism in the offspring. PTU was added to the drinking water from gestation day 6 to PND 21. Analysis of hippocampal BDNF levels, and learning and memory tests were performed on PNDs 45-52 on pups. These results indicated that hypothyroidism during the fetal period and the early postnatal period was associated with: (i) ~ 70% reduction of total serum T4, (ii) ~ 5-fold increase of total serum TSH levels (on PND 21; no significant differences could be found at the end of the behavioral testing, on PND 52), (iii) reduction of hippocampal BDNF protein levels (~ 8% decrease vs control), (iv) impairment of spatial learning and memory, in both male and female rat offspring.

- Gilbert et al., 2016: in this *in vivo* study, 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 and Sui, 2006: in this *in vivo* study, 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, 2011: in this *in vivo* study, trace fear conditioning deficits to context and to cue were reported in animals treated with PTU; animals 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.

In addition, studies *in humans* have shown associations between hypothyroidism and decreased memory and learning:

- Oerbeck et al., 2003: This study reported visual and verbal memory deficits in congenitally hypothyroid (CH) children. The CH group attained significantly lower scores than control subjects on intellectual, motor, and school-associated tests (total IQ: 102.4 vs 111.4). All verbal memory tasks were impaired; visual memory domain gave less consistent findings.

- Wheeler et al., 2011: Functional magnetic resonance imaging (fMRI) data from humans support the relationship between decreased serum concentrations of TH (occurring as a consequence of a decrease of TH synthesis) and memory deficits. CH subjects scored significantly below controls on indices of verbal but not visual memory as well as aspects of everyday memory functioning.

- Willoughby et al., 2013: The present study analysed 26 children with early-treated congenital hypothyroidism (CH), 23 children born to women with inadequately treated hypothyroidism during pregnancy (HYPO), and 30 typically developing controls. Results showed that relative to controls, CH and HYPO groups both exhibited weaknesses in episodic autobiographical memory, but not semantic autobiographical memory. In particular, CH and HYPO groups showed difficulty in recalling event details (i.e., the main happenings) and visual details from past experiences, confirming memory deficits.

- Willoughby et al., 2014: Congenitally hypothyroid children and children born to women with high TSH during pregnancy (biomarker of decreased TH synthesis) were weaker in recalling event and perceptual details from past naturally-occurring autobiographical events than age matched controls. HYPO cases scored significantly below controls on one objective and several subjective memory indices, and these were correlated with lower hippocampal volumes (brain region critical for learning and memory).

Uncertainties and Inconsistencies

Numerous studies reported that iodine deficiency in critical periods of brain development and growth causes severe and permanent growth and cognitive impairment (cretinism) (Pesce and Kopp, 2014; de Escobar et al., 2007; de Escobar et al., 2008; Zimmermann, 2007; Melse-Boonstra and Jaiswal, 2010; Horn and Heuer, 2010; Zimmermann, 2012). However, direct quantitative correlation between decreased TH synthesis (as a consequence of TPO inhibition) and decreased cognition, in support to this KER, were not assessed in these reports.

Moreover, Wheeler et al., 2012 used fMRI visuospatial memory task to assess hippocampal activation in adolescents with CH (N = 14; age range, 11.5-14.7 years) compared with controls (N = 15; age range, 11.2-15.5 years). Despite, adolescents with congenital hypothyroidism showed both increased magnitude of hippocampal activation relative to controls and bilateral hippocampal activation when only the left was observed in controls, no group differences were recorded in task performance.

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Relationship: 1504: TH synthesis, Decreased leads to BDNF, Reduced (<https://aopwiki.org/relationships/1504>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	non-adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

The connection between synthesis of TH and BDNF expression has been studied only in rodent models up to date.

Key Event Relationship Description

Several studies have shown that THs regulate BDNF expression in the brain (Koibuchi et al., 1999; Koibuchi and Chin, 2000; Sui and Li, 2010), with the subsequent neurodevelopmental consequences, as described in the direct KER. For example, during the early cortical network development TH has been shown to regulate the morphology and function of the GABAergic neurons (Westerholz et al., 2010) and BDNF is one of the mediators of this regulation (Binder and Scharfman, 2004; Gilbert and Lasley, 2013).

In view of the above evidence, it has been suggested that the thyroid insufficiency triggered by inhibition of TPO or NIS functions, resulting in decreased TH synthesis and subsequent lowered TH levels in serum and brain, may lead to reduction of the levels of BDNF mRNA or protein in the developmental brain.

Evidence Supporting this KER

Biological Plausibility

The importance of TH in brain development has been recognised and investigated for many decades (Bernal, 2011; Williams 2008). Several human studies have shown that low levels of circulating maternal TH, even in the modest degree, can lead to neurophysiological deficits in the offspring, including learning and memory deficits, or even cretinism in most severe cases (Zoeller and Rovet, 2004; Henrichs et al., 2010). The levels of serum TH at birth are not always informative, as most of the neurological deficits are present despite the normal thyroid status of the newborn. That means that the cause of these impairments is rooted in the early stages of the neuronal development during the gestational period. The nature and the temporal occurrence of these defects suggest that TH may exert their effects through the neurotrophins, as they are the main regulators of neuronal system development (Lu and Figurov, 1997). Among them, BDNF represents the prime candidate because of its critical role in CNS development and its ability to regulate synaptic transmission, dendritic structure and synaptic plasticity in adulthood (Binder and Scharfman, 2004). Additionally, hippocampus and neocortex are two of the regions characterized by the highest BDNF expression (Kawamoto et al., 1996), and are also key brain areas for learning and memory functions.

Empirical Evidence

Many in vivo studies have focused on the determination of the relationship between TH-mediated effects and BDNF expression in the brain. The majority of the work has been performed by evaluating the effects of TH insufficiency on BDNF developmental expression profile. The results, despite some differences, are showing a trend toward BDNF down-regulation triggered by decrease of TH synthesis.

Reductions in BDNF mRNA and protein were observed in hypothyroid rat models exposed to the TPO inhibitors methimazole (MMI) or propylthiouracil (PTU), and perchlorate (NIS inhibitor) (Koibuchi et al., 1999; 2001; Sinha et al., 2009; Neveu and Arenas, 1996; Lasley and Gilbert, 2011). These studies supported direct associations between decreased levels of TH and reduced BDNF expression in the developmental cerebellum, hippocampus and cortex. The dose-response relationship could not be evaluated in these studies, as they were conducted in conditions of severe maternal hypothyroidism, namely after exposure to very high doses of the chemicals.

- **Koibuchi et al., 2001:** In this in vivo study, newborn mice were rendered hypothyroid by administering MMI (TPO inhibitor) and perchlorate (NIS inhibitor) in drinking water to their mothers. Neurotrophin-3 (NT-3) and BDNF gene expression was depressed in the perinatal hypothyroid cerebellum. Furthermore, the expression of retinoid-receptor-related orphan nuclear receptor-alpha (ROR-alpha), an orphan nuclear receptor that plays critical roles in Purkinje cell development, was also decreased. Morphologically, disappearance of the external granule cell layer was retarded and arborization of Purkinje cell dendrite was decreased, events that were also observed in hypothyroid rats, suggesting impairment in neuronal differentiation.

- **Chakraborty et al., 2012:** In this in vivo study, PTU (TPO inhibitor) exposure in rat dams (4 ppm in drinking water) significantly decreased the levels of free T4 (~ 33% decrease vs control, at PND 7) and total T4 (~ 38% decrease vs control, at PND 7) in the offspring, and hippocampal BDNF protein levels in the offspring at 3 and 7 PNDs (~ 25% decrease of hippocampal BDNF, at PND 7, in female pups vs untreated female control). No significant BDNF reductions were observed in either the cerebellum or brain stem.

- **Blanco et al., 2013:** In this in vivo study, a significant dose-dependent down-regulation of hippocampal BDNF mRNA (~ 32% decrease vs control) in combination with the dose-dependent reduction of plasma TH (T4: ~ 25% decrease vs control; T3: ~ 14% decrease vs control), was also shown in Sprague Dawley rats after exposure to BDE-99 (2 mg/kg/day, through gavage, from gestation day 6 to PND 21).

- **Shafiee et al., 2016:** This in vivo study investigated the effects of PTU (TPO inhibitor, 100 mg/L) in pregnant rats to evaluate the effects elicited by maternal hypothyroidism in the offspring. PTU was added to the drinking water from gestation day 6 to PND 21. Analysis of hippocampal BDNF levels, and learning and memory tests were performed on PNDs 45-52 on pups. These results indicated that hypothyroidism during the fetal period and the early postnatal period was associated with: (i) ~ 70% reduction of total serum T4, (ii) ~ 5-fold increase of total serum TSH levels (on PND 21; no significant differences could be found at the end of the behavioral testing, on PND 52), (iii) reduction of hippocampal BDNF protein levels (~ 8% decrease vs control), (iv) impairment of spatial learning and memory, in both male and female rat offspring.

- **Gilbert et al., 2016:** In this in vivo study, developmental PTU treatment dramatically reduced the activity-dependent expression of neurotrophins and related genes in neonate hippocampus (e.g., at PND14, rats treated with 3 ppm PTU, underwent reduction of Bdnf (~25%, Bdnfiv (~10%, Ngf (~25%, and Pval (~50%) 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).

- **Abdelhaffez and Hassan, 2013:** This in vivo study in rats reported that methimazole (MMI, a TPO inhibitor)-induced hypothyroidism reduced plasma free T3, free T4 and significantly increased TSH in the pups, showing also reduced hippocampal and cerebellar BDNF levels.

- **da Conceição et al., 2016:** In this in vivo study, thyroidectomized (i.e., hypothyroid) adult Wistar rats showed significant increase of serum TSH (~ 750% increase vs control rats), decrease of T4 (~ 80% decrease vs control) and T3 serum levels (~ 45% decrease vs control), together with a reduced hippocampal expression of MCT8 (~ 83% decrease vs control rats), TH receptor alfa (TR α 1) (~ 77% decrease vs control), deiodinase type 2 (DIO2) (~ 90% decrease vs control), and BDNF mRNA expression in hippocampus (~ 75% decrease vs control).

- **Cortés et al., 2012:** In this in vivo study, adult male Sprague-Dawley rats were treated with 6-propyl-2-thiouracil (PTU, a TPO inhibitor) (0.05% in drinking water) for 20 days to induce hypothyroidism. PTU-treated rats showed decrease serum T4 (~ 70% decrease vs control) and tT3 (~ 45% decrease vs control) levels, and increased TSH levels (~ 9.5-fold increase over control). The hippocampus of hypothyroid adult rats displayed increased apoptosis levels in neurons and astrocytes and reactive gliosis compared with controls. The glutamatergic synapses from the stratum radiatum of CA3 from hypothyroid rats, contained lower postsynaptic density (PSD) than control rats (~ 25% lower PSD than control). This observation was in agreement with a reduced content of NMDAR subunits (NR1 and NR2A/B subunits, both subunits: ~ 25% decrease vs control) at the PSD in hypothyroid animals. Additionally, the hippocampal amount of BDNF mRNA (assessed by *in situ* hybridization) was higher (~ 4.8-fold increase over control) of hypothyroid rats, while the content of TrkB protein (BDNF receptor) was reduced (~ 30% decrease vs control) at the PSD of the CA3 region of hypothyroid rats, compared with controls. Even though BDNF levels were increased, the decrease of BDNF receptor (TrkB) compromises the signalling pathway under BDNF control.

- **Jang et al., 2012:** In this in vivo 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. 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 and cognitive function in future generations.

- **Pathak et al., 2011:** In this in vivo study, the effect of maternal TH deficiency on neocortical development was investigated. Rat dams were maintained on MMI (TPO inhibitor) from GD6 until sacrifice. Decreased number and length of radial glia, loss of neuronal bipolarity, and impaired neuronal migration were recovered with early TH replacement (at E13-15). BDNF mRNA resulted downregulated (80% decrease at E14) while TrkB expression was increased (2-fold) in hypothyroid fetuses at E14 stage. TH levels in the brain were not measured in this study.

- **Neveu and Arenas, 1996:** This in vivo study found that early hypothyroidism (by PTU administration to rat dams) decreased the expression of neurotrophin 3 (NT-3) and BDNF mRNA (70% reduction in BDNF level at P30). Grafting of P3 hypothyroid rats with cell lines expressing high levels of NT-3 or BDNF prevented hypothyroidism-induced cell death in neurons of the internal granule cell layer at P15.

Uncertainties and Inconsistencies

Despite the fact that many in vivo studies have shown a correlation between hypothyroidism and decreased BDNF expression in the brain, no clear consensus can be reached by the overall evaluation of the existing data. There are numerous conflicting studies showing no significant alterations in BDNF mRNA or protein levels (Alvarez-Dolado et al., 1994; Bastian et al., 2010; 2012; Royland et al., 2008; Lasley and Gilbert, 2011). However, the results of these studies cannot exclude the possibility of temporal- or region-specific BDNF effects as a consequence of foetal hypothyroidism. A transient TH-dependent BDNF reduction in early postnatal life can be followed by a period of normal BDNF levels or, on the contrary, normal BDNF expression in the early developmental stages is not predictive of equally normal BDNF expression throughout development. Moreover, significant differences in study design, the assessed brain regions, the age and the method of assessment in the existing studies, further complicate result interpretation.

While PTU (TPO inhibitor) has been shown to decrease brain BDNF levels and expression in offspring born from PTU-treated rat dams (Shafiee et al. 2016; Chakraborty et al., 2012; Gilbert et al. 2016), in Cortés et al., 2012 study (in vivo), treatment of adult male Sprague-Dawley rats with PTU induced an increase in the amount of BDNF mRNA in the hippocampus, while the content of TrkB, the receptor for BDNF, resulted reduced at the postsynaptic density (PSD) of the CA3 region compared with controls. Treated rats presented also thinner PSD than control rats, and a reduced content of NMDAR subunits (NR1 and NR2A/B subunits) at the PSD in hypothyroid animals. These indicate differential effects elicited by PTU (i.e., TPO inhibition) on BDNF expression/regulation comparing the adult vs foetal brain. However, even though BDNF levels were increased, the decrease of BDNF receptor (TrkB) compromises the signalling pathway under BDNF control.

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Relationship: 1505: TH synthesis, Decreased leads to GABAergic interneurons, Decreased (<https://aopwiki.org/relationships/1505>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	non-adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Moderate

Sex Applicability

Sex	Evidence
Male	
Unspecific	Moderate

Empirical evidence comes from work with laboratory rodents (rats and mice).

Key Event Relationship Description

Thyroid hormone synthesis is responsible for physiological TH serum levels that subsequently correlate with TH brain concentrations. It has been shown that TH regulates function of different neuronal subtypes, including GABAergic neurons. TH increases glutamic acid decarboxylase (GAD) activity (responsible for GABA-synthesis) in neonatal brain, and GABA transaminase (responsible for GABA degradation) activity (Shulga and Rivera, 2013). GABAergic interneurons are remarkably diverse and complex in nature and they are believed to play a key role in numerous neurodevelopmental processes (Southwell et al., 2014). During the early cortical network development TH has been shown to regulate the morphology and function of the GABAergic neurons (Westerholz et al., 2010). It is well documented that decreased TH synthesis triggered by TPO and NIS inhibitors affects survival of GABAergic interneurons, as well as their morphology and function.

Evidence Supporting this KER

Biological Plausibility

TH levels influence the development of cortical GABAergic circuits (Friau et al., 2008; Westerholz et al., 2010). In hypothyroid rats the expression of parvalbumin, the marker of a subpopulation of GABAergic neurons, is reduced (Gilbert et al., 2007). TH increase glutamic acid decarboxylase (GAD, GABA-synthesizing enzyme) activity in neonatal brain. Also GABA transaminase (GABA-T, GABA-degrading enzyme) activity appears to be increased by TH. Therefore, both GABA synthesis and degradation are increased by TH. This might reflect either the specific regulation of GABA levels, or general regulation of gene expression maintenance by TH, as commented by Shulga and Rivera, 2013. This strongly supports the link between the two KEs described in this indirect KER (decrease of TH synthesis leads to GABAergic interneuron decrease). It was also shown that low concentrations of T3 increase by non-genomic mechanism the depolarization-dependent release of GABA. GABA appears to provide negative feedback to thyroid endocrine axis, as TSH release is inhibited by GABA (Wiens and Trudeau, 2006).

Empirical Evidence

TPO inhibitors (like PTU and MMI) decrease the volume of the granule cell layer of the dentate gyrus, the density of cells within the layer, and estimates of total granule cell number, as shown in hypothyroid rats (Madeira et al., 1991). Migration of granule cells from the proliferative zone to the granule cell layer (with different neuronal subtypes, including GABAergic neurons) is retarded by thyroid deficiency, as is dendritic arborization and synaptogenesis assessed by immunohistochemistry for the synaptic protein synaptophysin (Rami et al., 1986a, 1986b, Rami and Rabie, 1990).

- Sawano et al., 2013: This *in vivo* study investigated the effects methimazole (MMI, a TPO inhibitor) on the developing rat hippocampus, one of the brain regions most sensitive to TH status. MMI was administered at the concentration of 0.025% in drinking water to pregnant dams from gestational day 15 until 4 weeks postpartum. Looking at the pre- and post-synaptic components of the GABAergic system, the level of glutamic acid decarboxylase 65 (GAD65) protein was reduced to less than 50% of control in the hippocampus of hypothyroid rats, and recovered to control levels by daily thyroxine-replacement after birth. Reduction in GAD65 protein was correlated immunohistochemically with a 37% reduction in the number of GAD65⁺ cells, as well as a reduction in GAD65⁺ processes. In contrast, GAD67 was not affected by MMI treatment. A subpopulation of GABAergic neurons containing PV was also confirmed to be highly dependent on TH status (with a 33% reduction in total PV⁺ neurons compared with the control). Moreover, the physiologically occurring transient rise of KCC2 expression observed at PND 10 (followed by a large increase in KCC2 protein at PND 15) in the euthyroid hippocampus, was completely suppressed by MMI (~ 80% reduction in KCC2 protein at PND 15 vs control).

- Shiraki et al., 2012: This *in vivo* study compared the differential effects of MMI (0, 50, 200 ppm in the drinking water) comparing the developmental and adult-stage, in particular comparing pregnant rats treated from gestation day 10 to PND 21 (i.e., developmental hypothyroidism) and adult male rats treated from PND 46 through to PND 77 (i.e., adult-stage hypothyroidism). With regard to precursor granule cells, a sustained reduction of Pax6⁺ stem or early progenitor cells and a transient reduction of doublecortin⁺ late-stage progenitor cells were observed after developmental hypothyroidism with MMI at 50 and 200 ppm. These cells were unchanged by adult-stage hypothyroidism. The number of PV⁺ cells (a GABAergic interneuron subpopulation in the dentate hilus) was decreased (~ 60% reduction at PND 21, with 200 ppm MMI) and the number of calretinin⁺ cells was increased (~ 85% increase at PND 21, with 200 ppm MMI) after both developmental and adult-stage hypothyroidism.

- Gilbert et al., 2007: In this *in vivo* study pregnant rat dams were exposed to propylthiouracil (PTU, a TPO inhibitor, administered at 0, 3, 10 ppm in the drinking water, from gestational day 6 until PND 30). PTU decreased maternal serum T4 by ~ 50-75% and increased TSH. At weaning, T4 was reduced by approximately 70% in offspring in the low-dose group and fell below detectable levels in high-dose animals. PV⁺ cells were diminished in the hippocampus and neocortex of offspring sacrificed on PND 21 (~ 45% reduction in the cortex, and ~ 55% reduction in the dentate gyrus, with 3 ppm treatment), and altered staining persisted to adulthood despite the return of TH to control levels.

- Westerholz et al., 2010; 2013: In the developing cortex, spontaneous activity is characterized by synchronous bursts of action potentials in populations of glutamatergic and GABAergic neurons which propagate throughout developing neural networks. In these *in vitro* studies with cortical neurons (prepared from E16 rat cortex), synthesis of TH and T3, in particular, increased the density and growth of GABAergic neurons and accelerated the maturation of neural networks.

Uncertainties and Inconsistencies

While some *in vivo* studies (Sawano et al., 2013; Shiraki et al., 2012) have shown a decrease of GABAergic cell populations upon induction of hypothyroidism, Saegusa and co-workers (Saegusa et al., 2010) reported about an increase of GABAergic interneurons. In Saegusa's study, rat dams were treated with either PTU or MMI in the drinking water, and male offspring were immunohistochemically examined on PND 20 and at the adult stage (i.e., 11-week-old). MMI and PTU caused in the offspring growth retardation, lasting into the adult stage. All exposure groups showed a sustained increase of GAD67⁺ cells in the adult stage, indicating an increase in GABAergic interneurons.

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It should be noticed that in Saegusa et al., 2010 in vivo study, increase of GAD67⁺ cells was mainly observed in the adult stage (11-week-old rats) and analysis of GABAergic interneurons. PV⁺ cells, which appear to be the GABAergic population most affected by TH dysregulation, was not evaluated. On the opposite, Sawano's and Shiraki's in vivo studies reported a decrease of GABAergic PV⁺ neurons at earlier stages, respectively on PND 15 and 21 induced by hypothyroidism (Sawano et al., 2013; Shiraki et al., 2012). Discrepancies in results are due to the fact that THs have effects on multiple components of the GABA system. For instance, in the developing brain, hypothyroidism generally decreases enzyme activities and GABA levels, whereas in adult brain, hypothyroidism generally increases enzyme activities and GABA levels.

There are also conflicting results on effects of long term changes in TH levels on GABA reuptake. Therefore, results variability from study to study is due to different experimental study designs, accounting for differences in brain development stages (PND vs adult), times of exposures to chemicals, and regional brain differences (Wiens and Trudeau, 2006).

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Relationship: 448: BDNF, Reduced leads to Synaptogenesis, Decreased (<https://aopwiki.org/relationships/448>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	non-adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	High

Sex Applicability

Sex	Evidence
Mixed	High

Empirical evidence comes from work with laboratory rodent-derived cells and brain slices, and rodent in vivo studies.

Key Event Relationship Description

Disruption of BDNF signaling (and other factors, such as NGF or Reelin, etc.) during brain development was shown to interfere with synaptogenesis in the hippocampus (Sanchez-Martin et al., 2013; Neal et al., 2010; Stansfiled et al., 2012). In the adult brain, BDNF is involved in synaptic plasticity (Lu et al., 2013; Leal et al., 2014), which is a fundamental process linked with learning and memory. Synaptic dysfunction is a key pathophysiological hallmark in neurodegenerative disorders, including Alzheimer's disease, and synaptic repair therapies based on the use of trophic factors, such as BDNF, are currently under consideration (Lu et al., 2013).

BDNF is released by the BDNF-producing neurons of the CNS and binds to TrkB of the PV-interneurons, an interaction necessary for the subsequent developmental effects of this neurotrophin (Polleux et al., 2002; Jin et al., 2003; Rico et al., 2002; Aguado et al., 2003). BDNF promotes the morphological and neurochemical maturation of hippocampal and neocortical interneurons and promotes GABAergic synaptogenesis (Danglot et al., 2006; Hu and Flussek, 2008).

BDNF plays an important role in axonal and dendritic differentiation during embryonic stages of neuronal development, as well as in the formation and maturation of dendritic spines during postnatal development (Chapleau et al., 2009). Recent studies have also implicated vesicular trafficking of BDNF via secretory vesicles, and both secretory and endosomal trafficking of vesicles containing synaptic proteins, such as neurotransmitter and neurotrophin receptors, in the regulation of axonal and dendritic differentiation, and in dendritic spine morphogenesis. Abnormalities in dendritic and synaptic structure

are consistently observed in human neurodevelopmental disorders associated with mental retardation, as well as in mouse models of these disorders (Chapleau et al., 2009).

Evidence Supporting this KER

Biological Plausibility

BDNF, in addition to its pro-survival effects, has powerful synaptic effects, promoting synaptic transmission, synaptic plasticity and synaptogenesis (Lu et al., 2013; Sanchez-Martin et al., 2013; Neal et al., 2010; Stansfield et al., 2012; Danglot et al., 2006; Hu and Russek, 2008). NMDAR activity has been linked to the signaling of the trans-synaptic neurotrophin BDNF (Neal et al., 2010).

Use of selective agonist or antagonist of BDNF receptor TrkB demonstrates the contribution of BDNF in synaptogenesis in adult-generated neurons in the rat dentate gyrus (Ambrogini et al., 2013). In this regard, exogenous application of BDNF significantly increased the number of functional synapses in culture (Vicario-Abejon et al., 1998; Marty et al., 2000), while blocking of BDNF with antibodies greatly reduced the formation of inhibitory synapses (Seil and Drake-Baumann, 2000). Similar results were described also in an *in vivo* study on mutant mice characterized by deletion of the *trkB* gene in cerebellar precursors (obtained by *Wnt1*-driven Cre-mediated recombination). TrkB mutant mice showed reduced amounts of GABAergic markers and develop reduced numbers of GABAergic boutons and synaptic specializations, whilst granule and Purkinje cell dendrites appeared normal and the former presented typical numbers of excitatory synapses. This study demonstrated that TrkB is essential to the development of GABAergic neurons and the regulation of synapse formation (Rico et al., 2002). BDNF is also a potent regulator of spontaneous neuronal activity in GABAergic neurons and interneurons, as shown in embryonic (E18) hippocampal slices (Aguado et al., 2003), and plays a critical role in controlling the emergence, complexity and networking properties of spontaneous networks.

TH deficiency during the foetal and/or the neonatal period, apart from reducing synaptogenesis, can produce several other deleterious effects for neural growth and development (e.g., such as reduced synaptic connectivity, delayed myelination, disturbed neuronal migration, deranged axonal projections, and alterations in neurotransmitters' levels), possibly through decreased BDNF levels (Koromilas et al., 2010; Shafiee et al., 2016).

Empirical Evidence

Several studies (*in vitro*, *ex vivo*, and *in vivo*) have shown correlations between downregulation of BDNF signaling (e.g., in transgenic animals, or upon treatment with K252a (a BDNF receptor inhibitor) or with an antibody anti-BDNF) and synaptogenesis (and synapses) decrease:

- **Westerholz et al., 2013** In recent *in vitro* studies with rat T3-deficient cultures of cortical GABAergic PV⁺ interneurons, which are subject to BDNF regulation, it was shown that the number of synaptic boutons (i.e., presynaptic terminals containing the presynaptic marker synaptophysin) was reduced, an effect that was abolished after exogenous BDNF application. Additionally, inhibition of BDNF TrkB receptors by K252a in cultures containing T3 resulted also in decreased number of synaptic boutons, as in the T3-deprived cultures. These results indicate that BDNF signaling promotes the formation of synaptic boutons and that this function is mediated by THs (T3 and T4). Additionally, T3-related increase of spontaneous network activity was remarkably reduced after addition of K252a, and also upon inhibition of mTOR pathway (with rapamycin), a pathway known to control synaptogenesis (Buckmaster et al., 2009).

- **Sato et al., 2007** This study on rat cultured hippocampal slices showed that beta-estradiol (E2) induced synaptogenesis between mossy fibers (one of the major inputs to cerebellum) and hippocampal CA3 neurons by enhancing BDNF release from dentate gyrus (DG) granule cells, by increasing the expression of PSD95, a postsynaptic marker. E2 effects on hippocampal slice cultures and subregional neuron cultures were completely inhibited by blocking the BDNF receptor (TrkB) with K252a (200 nM) or by using a function-blocking antibody to BDNF (10 µg/ml), which inhibited the expression of PSD95 induced by E2. Both K252a and the antibody anti-BDNF elicited ~ 60-70% decrease of spine density and ~ 55% decrease of presynaptic sites in dentate gyrus granule cells (measured as number of puncta/neuron).

- **Schjettan and Escobar, 2012** In this study, intrahippocampal microinfusion of BDNF (3 µg/3 µl; 0.2 µl/min, in adult rats modified the ability of the hippocampal mossy fiber pathway to present long-term potentiation (LTP, i.e., a persistent strengthening of synapses based on recent patterns of activity) by high frequency stimulation (HFS). This indicates that BDNF initiates the metaplastic mechanisms that allow the modifications of the ability of the mossy fiber pathway to present LTP induced by subsequent HFS. On the contrary, microinfusion of K252a (administered in combination with BDNF: 3 µg of BDNF/3 µl of K252a 20 µM; 0.2 µl/min) blocked the functional and morphological effects produced by BDNF (shown by densitometric analysis on synaptic reorganization: ~ 30% reduction of the relative area of the dorsal hippocampus in the contralateral side of HFS, and ~ 70% reduction in the ipsilateral side of HFS, compared to BDNF administered alone), supporting the role of BDNF in the regulation of synaptic plasticity.

- **Schildt et al., 2013** Using field potential recordings in CA3 of adult heterozygous BDNF knockout (BDNF+/-) mice, an impairment of NMDAR-independent mossy fiber (MF)-LTP (~ 50% decrease) was observed. Additionally, inhibition of TrkB/BDNF with K252a (slices preincubated for 3 hr with 100 nM), or with the selective BDNF scavenger TrkB-Fc (slices preincubated for 3 hr with 5 µg/ml), both inhibited MF-LTP to the same extent as observed in BDNF+/- mice (K252a: ~ 60% decrease vs control slices; TrkB-Fc: ~ 50% decrease vs control slices).

- **Cortés et al., 2012** Adult male Sprague-Dawley rats were treated with 6-propyl-2-thiouracil (PTU, a TPO inhibitor) (0.05% in drinking water) for 20 days to induce hypothyroidism. PTU-treated rats showed decrease serum fT4 (~ 70% decrease vs control) and tT3 (~ 45% decrease vs control) levels, and increased TSH levels (~ 9.5-fold increase over control). The hippocampus of hypothyroid adult rats displayed increased apoptosis levels in neurons and astrocyte and reactive gliosis compared with controls. The glutamatergic synapses from the stratum radiatum of CA3 from hypothyroid rats, contained lower postsynaptic density (PSD) than control rats (~ 25% lower PSD than control). This observation was in agreement with a reduced content of NMDAR subunits (NR1 and NR2A/B subunits, both subunits: ~ 25% decrease vs control) at the PSD in hypothyroid animals. Additionally, the hippocampal amount of BDNF mRNA (assessed by *in situ* hybridization) was higher (~ 4.8-fold increase over control) of hypothyroid rats, while the content of TrkB protein (BDNF receptor) was reduced (~ 30% decrease vs control) at the PSD of the CA3 region of hypothyroid rats, compared with controls. Even though BDNF levels were increased, the decrease of BDNF receptor (TrkB) compromises the signalling pathway under BDNF control.

- **Koibuchi et al., 2001** Here newborn mice were rendered hypothyroid by administering MMI (TPO inhibitor) and perchlorate (NIS inhibitor) in drinking water to their mothers. Neurotrophin-3 (NT-3) and BDNF gene expression was depressed in the perinatal hypothyroid cerebellum. Furthermore, the expression of retinoid-receptor-related orphan nuclear hormone receptor-alpha (ROR-alpha), an orphan nuclear receptor that plays critical roles in Purkinje cell development, was also decreased. Morphologically, disappearance of the external granule cell layer was retarded and arborization of Purkinje cell dendrite was decreased in hypothyroid rats. Dendritic arborization is used as readout for synapse formation, as post-synaptic side (synaptogenesis) is mainly located on dendrites.

- **Aguado et al., 2003** BDNF overexpression in transgenic embryos raised the spontaneous activity of E18 hippocampal neurons, as shown by increased number of synapses (63% more synapses in the hippocampus of BDNF transgenic embryos than in controls), and increased spontaneous neuronal activity (2.3 times more active neurons than wild type embryos, and 36.3% greater rates of activation). Moreover, BDNF transgenic embryos had higher number of GABAergic interneuron synapses, as shown by higher GAD67 mRNA (by 3-fold) and K(+)/Cl(-) KCC2 mRNA expression (by 4.3-fold) (responsible for the conversion of GABA responses from depolarizing to inhibitory), without altering the expression of GABA and glutamate ionotropic receptors. These data indicate that BDNF controls both GABAergic pre- and postsynaptic sites.

KEs proceeding the AO (decreased cognition), such as "Reduced BDNF Release" and "Decreased synaptogenesis" are 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 a reference chemical, are reported. These studies do not refer to TH disruption; however, they provide empirical support for this KER (Reduced release of BDNF leads to decreased synaptogenesis).

Synaptic structural plasticity was shown to be modified by Pb treatment during early (pre-weaning) or late (post-weaning) brain development in rats exposed to 2 mM Pb in drinking water for 3 weeks (Xiao et al., 2014). An iron chelator (cloquiquinol) can rescue the Pb-induced impairment of synaptic plasticity in hippocampus (Chen et al., 2007), showing that Pb can affect synaptogenesis and synaptic plasticity. Primary hippocampal neurons obtained from ED18 rat pups and treated with Pb (1, 2 microM) for 5 days exhibited pre-synaptic deficits due to disruption of NMDAR-dependent BDNF signaling (Neal et al., 2010; Stansfield et al., 2012). A decrease in bdnf expression was observed in mouse embryonic stem cells differentiated into neurons, if they were exposed to Pb 0.1 microM throughout the whole differentiation process (Sanchez-Martin et al., 2013). Similar alterations in gene expression patterns of neural markers (synapsin 1), neurotrophins (bdnf), transcription factors and glutamate-related genes were found in mice, when their mothers were exposed to 0.3 ppm of Pb in drinking water from 8 weeks prior to mating, through gestation and until postnatal day 10 (Sanchez-Martin et al., 2013).

Uncertainties and Inconsistencies

Alterations of BDNF signaling is probably not the only mechanism leading to impaired synaptogenesis and synaptic plasticity. Indeed NMDAR activity can also modulate nitric oxide (NO) signaling. Exogenous NO addition during Pb exposure results in complete recovery of whole-cell synaptophysin levels and partial recovery of synaptophysin and synaptobrevin in synapses in Pb-exposed neurons (Neal et al., 2012). In addition, in Wistar rats, the anti-oxidant and radical scavenger quercetin was able to relieve the impairment of synaptic plasticity induced by chronic Pb exposure (from parturition through adulthood (PND 60); 0.2% Pb in drinking water of mothers and post-weaning pups) (Hu et al., 2008), suggesting that oxidative stress can also interfere with synapse formation.

Additionally, while PTU (a TPO inhibitor) has been shown to decrease brain BDNF levels and expression in offspring born from PTU-treated rat dams (Shafiee et al. 2016; Chakraborty et al., 2012; Gilbert et al. 2016), in the study from Cortés and colleagues (Cortés et al., 2012), treatment of adult male Sprague-Dawley rats with PTU induced an increase in the amount of BDNF mRNA in the hippocampus, while the content of TrkB protein, the BDNF receptor, resulted reduced at the PSD of the CA3 region compared with controls. Treated rats presented also thinner PSD than control rats, and a reduced content of NMDAr subunits (NR1 and NR2A/B subunits) at the PSD. These indicate differential effects elicited by PTU (i.e., TPO inhibition) on BDNF expression/regulation comparing the adult vs foetal brain. However, even though BDNF levels were increased, the decrease of BDNF receptor (TrkB) compromises the signalling pathway under BDNF control.

Results variability from study to study is due to different experimental study designs, accounting for differences in brain development stages (PND vs adult), times of exposures to chemicals, and regional brain differences.

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Relationship: 1507: BDNF, Reduced leads to Impairment, Learning and memory (<https://aopwiki.org/relationships/1507>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Na⁺/I⁻ symporter (NIS) leads to learning and memory impairment (https://aopwiki.org/aops/54)	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Empirical evidence comes from *in vivo* studies with rodents.

Key Event Relationship Description

BDNF and its high-affinity receptor TrkB are widely expressed in the mammalian brain (Lewin and Barde, 1996). They play a crucial role in the development, maintenance and functioning of the CNS (Huang and Reichardt, 2003; Shafiee et al., 2016). BDNF is known to be directly regulated by thyroid hormones and plays essential roles during the critical period of fetal brain development (Wang et al., 2006), including cell proliferation, migration, differentiation, synaptogenesis and neuronal network formation. In addition, neuronal activity regulates BDNF transcription, transport of BDNF mRNA and protein into dendrites and the activity-dependent secretion of BDNF, which, in turn, modulate synaptic plasticity, synaptogenesis and memory formation (Bekinschtein et al., 2008).

Developmental thyroid hormone insufficiency is associated with reduced cognitive functions and lowered BDNF levels, as shown in both humans and animal models (Chakraborty et al., 2012). For instance, in rats, maternal thyroidectomy significantly reduces BDNF expression in the brain of developing pups (Liu et al., 2010), leading to learning and memory deficits. Prenatal exposure to PTU also leads to reduced hippocampal BDNF in neonatal rats (Chakraborty et al., 2012). This evidence supports the link between decrease of BDNF and learning and memory impairment described in this indirect KER.

Evidence Supporting this KER

Biological Plausibility

The BDNF gene is a key signal transduction element required for synaptic plasticity and many forms of associative learning (Lu et al., 2005; Park et al., 2013). Moreover, reduced function of BDNF leads to neurodevelopmental and learning disorders (Bienvenu et al., 2006). BDNF plays an important role in axonal and dendritic differentiation during embryonic stages of neuronal development, as well as in the formation and maturation of dendritic spines during postnatal development (Chapleau et al., 2009). Recent studies have also implicated vesicular trafficking of BDNF via secretory vesicles, and both secretory and endosomal trafficking of vesicles containing synaptic proteins, such as neurotransmitter and neurotrophin receptors, in the regulation of axonal and dendritic differentiation, and in dendrite spine morphogenesis. Abnormalities in dendrite and synaptic structure are consistently observed in human neurodevelopmental disorders associated with mental retardation, as well as in mouse models of these disorders (Chapleau et al., 2009).

BDNF protein is synthesized as a precursor (pre-proBDNF), resulting after cleavage in a 32-kDa proBDNF protein. ProBDNF is either proteolytically cleaved intracellularly by enzymes like furin or pro-convertingases and secreted as the 14-kDa mature BDNF (mBDNF), or secreted as proBDNF and then cleaved by extracellular proteases, such as metalloproteinases and plasmin, to mBDNF (see Lessmann et al., 2003). Both proBDNF and mBDNF are preferentially sorted and packaged into vesicles of the activity-regulated secretory pathway. ProBDNF is not an inactive precursor of BDNF; it is released in the immature and mature CNS in an activity dependent manner (for a comprehensive review on the role of BDNF in learning and memory, see Cunha et al. 2010). The intracellular localization of BDNF is predominantly somatodendritic, but it is also enriched in the dendrites. BDNF can activate several signalling pathways (e.g., ERK (Orban et al., 1999; Sweatt, 2004; Thomas and Huganir, 2004), PI3K–Akt (Lin et al., 2001), CREB (Barco et al., 2003)) that may regulate downstream cellular effects necessary for synaptic plasticity and memory formation. The role of BDNF in synaptogenesis and neuronal network functions, which represent the KEs before the AO (decrease of learning and memory), was already described in other three AOPs (i.e., 13, 48 and 12) already endorsed by OECD.

Importantly, reduced levels of BDNF have been reported as a consequence of decreased TH levels, playing a crucial role in neuroplasticity, one of the fundamental processes in learning and memory (Chakraborty et al., 2012; Gilbert and Lasley, 2013). In line with this, BDNF-mediated stimulation of both hippocampal neurogenesis and inhibition of hippocampal apoptosis can recover spatial memory deficits triggered by developmental hypothyroidism in rats (Shafiee et al., 2016; Shin et al., 2013).

Empirical Evidence

Some studies have shown associations between decrease of BDNF (e.g., as a consequence of exposure to several pollutants, such as BPA) and reduction of memory and learning:

- **Wang et al., 2016:** in this *in vivo* study, pregnant Sprague-Dawley female rats were orally treated with either vehicle or BPA (0.05, 0.5, 5 or 50 mg/kg BW/day) during days 9-20 of gestation. Male offspring were tested on PND 21 with the object recognition task. Data revealed a decrease in BDNF (~ 38% decrease at 50 mg/kg BW/day vs control) in the hippocampus. BPA-exposed male offspring underwent memory and cognitive impairments: they not only spent more time (~ 43% more, at 1.5 hr after training) in exploring the familiar object at the highest dose than the control, but also displayed a significant decrease in the object recognition index (at 50 mg/kg BW/day, ~ 54% lower short term memory measured 1.5 hr after training).

- **Jang et al., 2012:** In this *in vivo* 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 (causing inhibition of NIS function) in pregnant mothers could decrease hippocampal neurogenesis and cognitive function in future generations.

- **Shafiee et al., 2016** This *in vivo* study investigated the effects of PTU (TPO inhibitor, 100 mg/L) in pregnant rats to evaluate the effects elicited by maternal hypothyroidism in the offspring. PTU was added to the drinking water from gestation day 6 to PND 21. Analysis of hippocampal BDNF levels, and learning and memory tests were performed on PNDs 45-52 on pups. These results indicated that hypothyroidism during the fetal period and the early postnatal period was associated with: (i) ~ 70% reduction of total serum T4, (ii) ~ 5-fold increase of total serum TSH levels (on PND 21; no significant differences could be found at the end of the behavioral testing, on PND 52), (iii) reduction of hippocampal BDNF protein levels (~ 8% decrease vs control), (iv) impairment of spatial learning and memory, in both male and female rat offspring.

- **Gilbert and Sui, 2006; Gilbert, 2011; Gilbert et al., 2016:** (in vivo studies) Long-term potentiation (LTP) is a model of activity-dependent synaptic plasticity critical for learning and memory. LTP is impaired in both sub-regions of hippocampus under conditions of TH deficiency, and these impairments persist in the adult offspring on recovery of euthyroid status. LTP induces activation of neurotrophins, particularly BDNF and related signaling molecules. Induction of these pathways underlies the persistence of experience-dependent plasticity. Offspring of hypothyroid animals are deficient in LTP and in the induction of neurotrophin gene changes in response to neuronal activation. Similar plasticity is operative during early brain development, influencing synapse formation and formation of neural networks.

- **Gilbert et al., 2016:** in this *in vivo* study, 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 (e.g., at PND14, rats treated with 3 ppm PTU, underwent reduction of Bdnf by ~25%, Bdnfiv by ~10%, Ngf by ~25%, and Pval by ~50%) 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).

- **Liu et al., 2010:** This *in vivo* 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). Data showed that rat pups born from subclinical hypothyroidism dams had lower BDNF mRNA expression (on PND 3) and protein level (on PND 3 and PND 7) in the hippocampus. Moreover, 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.

- **Wang et al., 2012:** This *in vivo* 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 both SCH and H groups had lower levels of BDNF protein (~35% lower in both groups) and also of Egr1, Arc, and p-ERK compared to controls. T4 treatment ameliorated these protein expression changes in the progeny of rats with subclinical hypothyroidism. Moreover, the 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 BDNF and learning and memory deficits.

- **Bekinschtein et al. 2008:** in this *in vivo* study, the protein synthesis inhibitor anisomycin (Ani; 80 µg/0.8 µl per side) was injected in the dorsal hippocampus of Male Wistar rats (2.5 months) 12 h after inhibitory avoidance (IA) training (i.e., using a strong foot shock, which generates a persistent LTM), which causes a selective deficit in memory retention 7 days, but not 2 days, after training. Human recombinant BDNF (hrBDNF, 0.25 µg/0.8 µl per side) or vehicle (Veh) was delivered 15 min after Ani infusion into the hippocampus. hrBDNF completely rescued long-term memories (LTM) at 7 days after training caused by Ani given at 12 h after training. Additionally, infusion of BDNF antisense oligonucleotides (i.e., BDNF ASO, which blocks the expression of BDNF 12 h after training) into the dorsal hippocampus 10 h after training, was found to impair persistence (a characteristic feature of LTM), but not formation of IA LTM (as compared with BDNF missense oligonucleotide). This indicates that BDNF during the late posttraining critical time period is not only required but sufficient for persistence of LTM storage. This study also supports essentiality of this KE (i.e., decreased BDNF).

- **Alonso et al. 2002:** in this *in vivo* study the role of BDNF in both short and long term memories (STM and LTM) formation of a hippocampal-dependent one-trial fear-motivated learning task was examined in male Wistar rats (2-3 months). IA training was found associated with a rapid and transient increase in BDNF mRNA expression (by 90%, 1 hr after IA training) in the hippocampus. Bilateral infusions of function-blocking anti-BDNF antibody (0.5 µg/side) into the CA1 region of the dorsal hippocampus decreased ERK2 activation, and blocked STM formation. On the contrary, intrahippocampal administration of rhBDNF (0.25 µg/side) increased ERK1/2 activation and facilitated STM. These results strongly indicate that endogenous BDNF is required for both STM and LTM formation of an IA learning. This study also supports essentiality of this KE (i.e., decreased BDNF).

- **Blanco et al. 2013:** in this *in vivo* study rat dams were exposed to 0, 1 and 2 mg/kg/day of BDE-99 from GD 6 to PND 21. Data showed that transmission of maternal accumulated BDE-99 through placenta and breast milk caused a decrease of serum levels of T3 (by 13 ± 9% in the 2 mg/kg/day group), T4 (by 25 ± 13% in the 2 mg/kg/day group) and a decrease of free-T4 (by up to 17 ± 9% in the 2 mg/kg/day group), causing downregulation of BDNF gene expression in the hippocampus of pups (by 32 ± 14% in the 2 mg/kg/day group). Moreover, BDE-99 produced a delay in the spatial learning task in the water maze test (i.e., longer latency in reaching the platform at the highest BDE-99 dose vs control group), and a dose-response anxiolytic effect as revealed by the open-field test.

Uncertainties and Inconsistencies

There are no inconsistencies in this KER; however, alterations of BDNF signalling is reliably not the only mechanism leading to impaired learning and memory. Additional studies are required to better correlate BDNF levels, TH brain levels with learning and memory tests performed simultaneously.

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