AOP ID and Title:

SNAPSHOT

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AOP 54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children

Short Title: NIS inhibition and DNT effects

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 brain development and cognitive function in mammals relays 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 form 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 hormone synthesis and secretion into blood leading to subsequent TH insufficiency in the brain with detrimental effects in neurocognitive function in children. The present AOP describes developmental neurotoxicity (DNT) effects induced by the decreased levels of TH in the blood and consequently in the brain, as a result of NIS inhibition. Many environmental chemicals have been reported to disrupt iodide uptake, but the studies that have been focused on NIS inhibition are mainly restricted to perchlorate and some small ionic or drug-like molecules. Perchlorate, which is the most potent inhibitor of NIS, has been associated with reduced TH production and also with cognitive deficits in animals and humans.

Summary of the AOP

Stressors

Name	Evidence
Perchlorate	Strong
Nitrate	Strong
Thiocyanate	Strong
Dysidenin	Strong
Aryltrifluoroborates	Moderate

Molecular Initiating Event

Title	Short name
Inhibition, Na+/I- symporter (NIS)	Inhibition, Na+/I- symporter (NIS)

424: Inhibition, Na+/I- symporter (NIS)

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

AOPs Including This Key Event

AOP ID and Name	Event Type
65: XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	MolecularInitiatingEvent
134: Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent
176: Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	MolecularInitiatingEvent

Stressors

Name

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

Evidence for Perturbation of this Molecular Initiating Event by Stressor

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). 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). Finally, ten more small simple-structured molecules were identified in a large screening study (Lecat-Guillet 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 μM on rat-derived thyroid cells (Lecat-Guillet et al., 2008a). The biological activity is rationalized by the presence of the BF_3^- ion as a minimal binding motif for substrate recognition at the iodide binding site.

Biological Organization

Level of Biological Organization
Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
Pig	Pig	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
Pregnancy	Moderate
Birth to <1 month	Moderate

Sex Applicability

Sex	Evidence
Mixed	Strong

Apart from the human, functional NIS protein has been also identified in 3 other species, namely the rat (Dai et al., 1996), the mouse (Perron et al., 2001) and the pig (Selmi-Ruby et al., 2003). 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 themouse orthologs were shown to accumulate radioisotopes more efficiently than the human protein (Dayem et al., 2008; Heltemes 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 KmI value determined for hNIS (9.0 ± 0.8 µM) was significantly lower than the KmI for the mouse protein (26.4 ± 3.5 µM) whereas the KmNa 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 proteinwhile 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).

How this Key Event Works

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 tha transport 2 sodium cations (Na+) for each iodide anion (I-) into the follicular thyroid cell (Dohan et al., 2003). It has been also shown that many other anions, such as ClO3-, SCN-, NO3-, ReO4-, TcO4- and in a lower extent Br- and BF4-, are 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-Guillet et al., 2008a). A library of 17,020 compounds was tested by a radioactive screening method with high specificity using transfected mammalian cells (Lecat-Guillet 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). 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 la., 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 (Bizhanova 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 temporaly 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 (SLCA5A) 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 rear 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. 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). The measurement of iodide uptake in this case is done with an indirect spectophotometric method by using FRTL5 cells. This assay is equally sensitive with the radioiodine detection method.

3. Additionally, a fluorescence-based method has been developed, which uses a variant of the Yellow Fluorescent Protein (YFP) in order to detect the efflux of iodide into the FRTL5 cells. 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).

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Key Events

Title	Short name

Decreased, Thyroidal iodide uptake	Decreased, Thyroidal iodide uptake
Decreased, Thyroxine (T4) in neuronal tissue	Decreased, Thyroxine (T4) in neuronal tissue
Reduced, Release of BDNF	Reduced, Release of BDNF
Decreased, Synaptogenesis	Decreased, Synaptogenesis
Decreased, Thyroxin (T4) in serum	Decreased, Thyroxin (T4) in serum
Altered, GABAergic interneurons morphology and function	Altered, GABAergic interneurons morphology and function
Decreased, Neuronal network function in developing brain	Decreased, Neuronal network function in developing brain
Decreased, Thyroid hormone synthesis	Decreased, Thyroid hormone synthesis

425: Decreased, Thyroidal iodide uptake

Short Name: Decreased, Thyroidal iodide uptake

AOPs Including This Key Event

AOP ID and Name	Event Type
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	KeyEvent
134: Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
176: Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
188: Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

rat	Rattus norvegicus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
Pig	Pig	Strong	NCBI
human	Homo sapiens	Strong	NCBI

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 dyshormonogenesis 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).

How this Key Event Works

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 bounds 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 bounding rate of iodide is 50-100% of the intrathyroidal 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 earth's 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 well-known iodine transporter protein (Jhiang et al., 1998; Cho et al., 2000). The thyroid, salivary glands and the gastric mucosa have a common embryologic origin, from the primitive alimentary tract, which may explain the reason of the NIS expression in these tissues. Furthermore, in regards to the gastric mucosa and the breast, there is an obvious value of concentrating iodide, as it is the route for its derivation to the bloodstream and to the breast milk, respectively. The iodide from the circulation will eventually reach the thyroid in order to participate in its most important function, namely the production of thyroid hormones. In contrast, the biological role of iodide in the salivary glands and the choroid plexus is not yet specified, but it is a research area of high interest, as it is believed that it may be involved in important pathways but yet undiscovered.

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 iodothyroglobulin 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. Although T3 is more biologically active than T4, the major production of T3 actually occurs outside of the thyroid gland. The majority of T3 is produced by peripheral conversion from T4 in a deiodination reaction involving a specific enzyme which removes one iodine from the outer ring of T4.

A sodium-iodide (Na/I) symporter pumps iodide (I−) 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 (I0) by an enzyme called thyroid peroxidase (TPO). Iodine (I0) 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. The major thyroid hormone binding protein is thyroxin binding globulin (TBG) which accounts for about 75% of the bound hormone. In order to attain normal levels of thyroid hormone synthesis, an adequate supply of iodine is essential. In iodine sufficient areas, 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 H_2O_2 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) and the subsequent formation of MIT and DIT (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 (Glinoer, 1997). Additionally, small iodine

concentrations have been found to have significant antioxidant effects that resembles to ascorbic acid (Smyth, 2003).

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. 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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280: Decreased, Thyroxine (T4) in neuronal tissue

Short Name: Decreased, Thyroxine (T4) in neuronal tissue

AOPs Including This Key Event

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

Biological Organization

Level of Biological Organization
Organ

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
chicken	Gallus gallus	Weak	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Moderate

THs are critical for normal brain development in most vertebrates, primarily documented empirically in mammalian species. 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).

How this Key Event Works

Thyroid hormones are present in brain tissue of most vertebrate species. The amount of THs in brain is known to vary during development and to differ amoung 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 D2. D2 serves to convert T4 to T3. During this time D3 activity remains very low in cortex, D3 serving to convert T3 to an inactive form, rT3. In contrast, in other brain regions including hippocampus and cerebellum, T3 remains low throughout early and mid-gestation and corresponds with high activity of D3 in these brain regions. In late gestation and after birth, D3 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 in the fetal rat brain making quantitification difficult. Brain T3 and T4 rise in parallel from gestational day 10 to gestational day 20 in rat. They are typically both quite low tuntil gestational 17 with steep increases between GD18 and GD20 corresponding to the onset of fetal thyroid function (Calvo et al., 1990; Ruiz de Ono 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 in in the form of T4, primarily thourhg the OATP1c1 transporter, and 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 (D2) (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 D3 (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., Morreale de Escobar, 1985; 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. Two analytical techniques, LC-tandom mass spectrometry (LC/MS-MS), and LC–inductively coupled plasma–mass spectrometry (LC–ICP–MS) have recently been used to measure brain concentrations of TH. The latter (Simon et al., 2002) has proven capable of measuring very low levels in whole-body homogenates of frog tadpoles at different developmental stages. 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-triidothyronine (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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381: Reduced, Release of BDNF

Short Name: Reduced, Release of BDNF

AOPs Including This Key Event

AOP ID and Name	Event Type

13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	KeyEvent
12: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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 et al., 2014; Heinrich and Pagtakhan, 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).

How this Key Event Works

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, 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. 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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385: Decreased, Synaptogenesis

Short Name: Decreased, Synaptogenesis

AOPs Including This Key Event

AOP ID and Name	Event Type

13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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).

How this Key Event Works

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 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 (VGLUTs) 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 gephryin (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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281: Decreased, Thyroxin (T4) in serum

Short Name: Decreased, Thyroxin (T4) in serum

AOPs Including This Key Event

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

176: Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
194: Hepatic nuclear receptor activation leading to altered amphibian metamorphosis	KeyEvent

Biological Organization

Level of Biological Organization
Tissue

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
African clawed frog	Xenopus laevis	Strong	NCBI
chicken	Gallus gallus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

The overall evidence supporting taxonomic applicability is strong. THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in amphibian and larbean 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 T3 and T4 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 in adult humans is about 75, 15 and 10 percent, respectively (Schussler 2000). In contrast, the majority of THs are bound to TTR in adult rats. And thyroid binding proteins are developmentally regulated in rats. Thyroxine binding globulin is expressed in rats until approximately postnatal day (PND) 60, with peak expression occurring during weaning (Savu et al., 1989). Low 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 hormone half-life (Capen, 1997) and possibly regulatory feedback mechanisms, there is little information on quantitative dose-response relationships. SerumTHs are still regarded as the most robusts measurable key event causally linked to downstream adverse outcomes.

How this Key Event Works

There are two biological active thyroid hormones (THs) in serum, triiodothyronine (T3) and thyroxine (T4), and a few inactive iodothyronines (rT3, 3,5-T2), which are all derived from the modification of tyrosine molecules (Zoeller et al., 2007). However, the plasma concentrations of the other iodothyronines are significantly lower than those of T3 and T4.

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 active and 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 (Zoeller et al., 2007).

In rodents, serum TH are low in the fetal circulation, increasing as the fetal thyroid gland becomes functional on gestational day 17, just prior to birth on gestational day 22. After birth serum hormones increase steadily, peaking at two weeks, and falling slightly to adult levels by postnatal day 21 (Zoeller et al., 2007).

How it is Measured or Detected

Serum T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone are considered more direct indicators of T4 and T3 activities in the body, but in animal studies, total T3 and T4 are typically measured as the concentrations of free hormone are very low and difficult to detect. Historically, the most widely used method in toxicology is radioaminoassay (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. Least common is analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates, though methods employing HLPC and mass spectrometry exist (Hornung et al., 2015; DeVito et al., 1999; Spencer, 2013).

Any of these measurements should be evaluated for fit-for-purpose, relationship to the actual endpoint of interest, repeatability, reproducibility, and lower limits of quantification. 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 of these methods, particularly RIA, are repeatable and reproducible.

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851: Altered, GABAergic interneurons morphology and function

Short Name: Altered, GABAergic interneurons morphology and function

AOPs Including This Key Event

AOP ID and Name	Event Type
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
Caenorhabditis elegans	Caenorhabditis elegans	Weak	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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).

How this Key Event Works

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) and they have common features to distinguish them from the pyramidal excitatory cells. These include aspiny dendrites and the release of GABA neurotransmitter, which makes them the main inhibitory source in the 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 in that projection neurons send their signals to more distant locations such as the brain or the spinal cord. 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). One of the milestones at the crucial 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 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). 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). GABA is the first excitatory transmitter and is crucial during embryogenesis as 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 GABA-mediated depolarizing effects at the post-synaptic neurons in early development are well described (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 relevance during the formation of connections within the network (Wang and Kriegstein, 2010; Ben Ari et al., 2007; Blankenship and Feller, 2010). Furthermore, GABA-mediated depolarisations have recently been shown to promote excitatory synapse formation by facilitating NMDA receptor activation in cortical pyramidal neurons (Wang and Kriegstein, 2008). The effects of depolarizing GABA 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

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; Gangulu et al., 2001; Baltz et al., 2010; Westerholz et al., 2013). Additionally, GABA-immunohistochemistry can be used for identification and morphometric analysis of the 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.

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386: Decreased, Neuronal network function in developing brain

Short Name: Decreased, Neuronal network function in developing brain

AOPs Including This Key Event

AOP ID and Name	Event Type
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
78: Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 1	KeyEvent
90: Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2	KeyEvent
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI

mice	Mus sp.	Strong	NCBI
cat	Felis catus	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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).

How this Key Event Works

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

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

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

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

much earlier. The “silent” synapses disappear by PND 7-8 in both brain regions mentioned above.

There is strong evidence suggesting that NMDA receptor subunit composition controls synaptogenesis and synapse stabilization (Gambrill and Barria, 2011). It is established fact that during early postnatal development in the rat hippocampus, synaptogenesis occurs in parallel with a developmental switch in the subunit composition of NMDA receptors from NR2B to NR2A. It is suggested that early expression of NR2A in organotypic hippocampal slices reduces the number of synapses and the volume and dynamics of spines. In contrast, overexpression of NR2B does not affect the normal number and growth of synapses. However, it does increase spine motility, adding and retracting spines at a higher rate. The C terminus of NR2B, and specifically its ability to bind CaMKII, is sufficient to allow proper synapse formation and maturation. Conversely, the C terminus of NR2A was sufficient to stop the development of synapse number and spine growth. These results indicate that the ratio of synaptic NR2B over NR2A controls spine motility and synaptogenesis, and suggest a structural role for the intracellular C terminus of NR2 in recruiting the signalling and scaffolding molecules necessary for proper synaptogenesis. Interestingly, it was found that genetic deletion of NR3A accelerates glutamatergic synaptic transmission, as measured by AMPAR-mediated postsynaptic currents recorded in hippocampal CA1. Consistent, the deletion of NR3A accelerates the expression of the glutamate receptor subunits NR1, NR2A, and GluR1 sugesting that glutamatergic synapse maturation is critically dependent upon activation of NMDA-type glutamate receptors (Henson et al., 2012).

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

How it is Measured or Detected

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

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

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

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277: Decreased, Thyroid hormone synthesis

Short Name: Decreased, Thyroid hormone synthesis

AOPs Including This Key Event

AOP ID and Name	Event Type
42: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental	KeyEvent

Outcomes in Mammals	
65: XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
128: Kidney dysfunction by decreased thyroid hormone	MolecularInitiatingEvent
134: Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	KeyEvent
159: Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation	KeyEvent
175: Thyroperoxidase inhibition leading to altered amphibian metamorphosis	KeyEvent
176: Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
188: Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	KeyEvent
192: Pendrin inhibition leading to altered amphibian metamorphosis	KeyEvent
193: Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Strong	NCBI
human	Homo sapiens	Strong	NCBI
Pig	Pig	Strong	NCBI
Xenopus laevis	Xenopus laevis	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Decreased TH synthesis resulting from TPO or NIS inhibition is conserved across taxa, with in vivo evidence from humans, rats, amphibians, 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.

Typically decreased serum thyroxine (T4) is used as a surrogate measure to indicate chemical-mediated decreases in TH synthesis. However, clinical and veterinary management of hyperthyroidism and Grave's disease involves administration of drugs including propylthiouracil and methimazole, known to decrease TH synthesis, indicating strong medical evidence for chemical initiation of this event (Zoeller and Crofton, 2005).

How this Key Event Works

Thyroid hormone (TH) synthesis 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 serum levels are controlled by an efficiently regulated feedback mechanism: the hypothalamus-pituitary-thyroid (HPT) axis. 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 cell 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 pro-hormone T4, and to a lesser extent of T3, the biologically active TH. Most of the hormone released into circulation is is in the form of T4, while peripheral deiodination of T4 is responsible for the majority of circulating T3. Outer ring deiodination of T4 is conducted by activation of the deiodinating enzymes D1 and D2 (Bianco et al., 2006), 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).

The majority of 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, especially early in fetal life. In humans, THs can be found in the fetus as early as gestational weeks 10-12, and concentations 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 around the third week of gestation. (see Howdeshell, 2002; Santisteban and Bernal, 2005 for review).

Decreased TH synthesis in the thyroid gland may result from one or a combination of a set of possible molecular-initiating events (MIEs) including: 1) Inhibition of TPO, inhibition of the NIS, or dietary iodine insufficiency. Theoretically, decreased synthesis of Tg could also affect TH production (Kessler et al., 2008; Yi et al., 1997). 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.

It should be noted that different species and different lifestages 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 Tg-DIT residues to supply for several months to a year of TH demand (Greer et al., 2002). 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 asthese enzymes are essential for TH synthesis. Rarely is decreased TH synthesis measured directly, but rather the impact of chemicals onthe quantity of T4 released from the thyroid gland is assessed (e.g., Romaldini et al., 1988). Methods used include, use 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 of the necessary synthesis enzymes including TPO and NIS (Hornung et al., 2010). For this work TH was measured by HPLC/ICP-mass spectometry. Decreased TH synthesis and release, using T4 release as the endpoint, has been shown for thiouracil antihyperthyroidism drugs including MMI, PTU, and the NIS inhibitor perchlorate (Hornung et al., 2010).

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Adverse Outcomes

Title	Short name
Impairment, Learning and memory	Impairment, Learning and memory

341: Impairment, Learning and memory

Short Name: Impairment, Learning and memory

AOPs Including This Key Event

AOP ID and Name	Event Type
13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	AdverseOutcome
48: Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	AdverseOutcome
54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children	AdverseOutcome
77: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 1	KeyEvent

78: Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 1	KeyEvent
87: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 2	KeyEvent
88: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 3	KeyEvent
89: Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure 4	KeyEvent
90: Nicotinic acetylcholine receptor activation contributes to abnormal roll change within the worker bee caste leading to colony loss/failure 2	KeyEvent
12: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	AdverseOutcome

Biological Organization

Level of Biological Organization
Individual

Evidence Supporting Applicability of this Event

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Learning and memory have been studied in invertebrates such as gastropod molluscs and drosophila and vertebrates such as rodents and primates. Recently, larval zebrafish has also been suggested as a model for the study of learning and memory (Roberts et al., 2013).

How this Key Event Works

Learning can be defined as the process by which new information is acquired to establish knowledge by systematic study or by trial and error (Ono, 2009). Two types of learning are considered in neurobehavioral studies: a) associative learning and b) non-associative learning.

Associative learning is learning by making associations between different events. In associative learning, a subject learns the relationship among two different stimuli or between the stimulus and the subject's behaviour. Classical conditioning, operant conditioning and category learning are some examples of associative learning. On the other hand, non-associative learning can be defined as an alteration in the behavioral response that occurs over time in response to a single type of stimulus. Habituation and sensitization are some examples of non-associative learning. Another important type of learning is emotional learning and the simplest form of emotional regulation is extinction (Quirk and Mueller, 2008). During extinction, conditioned response to a stimulus decreases when the reinforcer is omitted and fear conditioning experiments help to elucidate the underlined mechanism.

The memory to be formed requires acquisition, retention and retrieval of information in the brain, which is characterised by the non-conscious recall of information (Ono, 2009). Memory is considered very important as it allows the subjects to access the past, to form experience and consequently to acquire skills for surviving purposes. There are three main categories of memory, including sensory memory, short-term or working memory (up to a few hours) and long-term memory (up to several days or even much longer). At the cellular level the storage of long-term memory is associated with increased gene expression and protein synthesis as well as formation of novel synaptic connections (Lynch, 2004).

Learning-related processes require neural networks to detect correlations between events in the environment and store these as changes in synaptic strength (Abbott and Nelson, 2000). Long-term potentiation (LTP) and long-term depression (LTD) are two fundamental processes involved in cognitive functions (Abbott and Nelson, 2000; Malenka and Bear, 2004), which respectively, strengthen synaptic inputs that are effective at depolarizing the postsynaptic neuron and weaken inputs that are not, thus reinforcing useful pathways in the brain. Synapses that are strengthened become more effective at depolarizing the postsynaptic neuron, eventually driving neuronal activity to saturation (Abbott and Nelson, 2000). As correlated activity of presynaptic and postsynaptic neurons drives strengthening of specific synapses, the postsynaptic neuron will be driven more strongly, and so presynaptic inputs that were initially only poorly correlated with postsynaptic firing will be better able to trigger firing of the postsynaptic neuron. This implies that nervous systems must have a matching set of plasticity mechanisms that counteract these destabilizing forces. The cortical and hippocampal pyramidal neurons have a target firing rate, and synaptic strengths are regulated to maintain these rates relatively constant in the face of perturbations in input channel (Burrone et al., 2002). This provides a robust mechanism for generating stability in network function in the face of learning-related changes in synaptic input. In principle, neurons could maintain stable firing rates through homeostatic regulation of many aspects of neuronal excitability. These possibilities include balancing inward and outward voltage-dependent conductances that determine firing properties generally called "intrinsic excitability" (Marder and Goaillard, 2006; Zhang and Linden 2003), regulating inhibitory and/or excitatory synaptic strength (Turrigiano, 2011) or synapse number (Kirov et al., 1999) or by adjusting the ease with which other forms of

plasticity can be induced, so-called “metaplasticity” (Abraham and Bear, 1996). Evidence suggests that all of these mechanisms can contribute to the homeostatic regulation of neuronal firing rates in central circuits. Activity-dependent alteration in synaptic strength is a fundamental property of the vertebrate central nervous system and is thought to underlie learning and memory.

A major expression mechanism of synaptic scaling is changes in the accumulation of synaptic glutamate receptors. Central synapses typically cluster both AMPA receptors and NMDA receptors. AMPA receptors are ionotropic and carry out the majority of excitatory synaptic current in the central nervous system; NMDA receptors are also ionotropic but open as a function of voltage, flux calcium, and mediate a number of calcium-dependent forms of synaptic plasticity (Malenka and Bear, 2004). Synaptic scaling results in postsynaptic changes in both types of glutamate receptors (Stellwagen and Malenka, 2006; Watt et al., 2000) and can therefore be monitored by measuring changes in receptor accumulation at synapses.

The best characterized form of LTP occurs in the CA1 region of the hippocampus, in which LTP is initiated by transient activation of receptors and is expressed as a persistent increase in synaptic transmission through AMPA receptors followed by activation of NMDARs. This increase is due, at least in part, to a postsynaptic modification of AMPA-receptor function; this modification could be caused by an increase in the number of receptors, their open probability, their kinetics or their single-channel conductance. Summing up activity-dependent alteration in synaptic strength is a fundamental property of the vertebrate central nervous system that underlies learning and memory processes.

It is appropriate to state that while much emphasis has been given on the key role of the hippocampus in memory, it would probably be simplistic to attribute memory deficits solely to hippocampal damage (Barker and Warburton, 2011). There is substantial evidence that fundamental memory functions are not mediated by hippocampus alone but require a network that includes, in addition to the hippocampus, anterior thalamic nuclei, mammillary bodies cortex, cerebellum and basal ganglia (Aggleton and Brown, 1999; Doya, 2000; Mitchell et al., 2002, Toscano and Guilarte, 2005). Each of these brain structures can be potentially damaged leading to more or less severe impairment of learning and memory.

Amnesia is defined as the impairment or loss of memory. Depending on the cause amnesia can be characterised as functional, organic amnesia or infantile amnesia. Dementia, is a brain disease that causes a long term and often gradual decrease in the ability to think and remember as well as problems with language, and a decrease in motivation (Solomon and Budson, 2011). It is an intellectual impairment observed mainly in elderly people due to the progress of a neudegenerative disease. In younger people this type of impairment is known as presenile dementia. The most common affected areas include memory, visual-spatial, language, attention, and executive function (problem solving). Therefore, very often, short-time memory, mind, speech and motor skills are affected. Certain forms of dementia can be treated, to some extent. The most common form of dementia is Alzheimer's disease, which accounts for between 50 and 60 percent of all cases. Other types include vascular dementia and Lewy body dementia (Burns, 2009). Initial symptoms in Alzheimer's disease is memory impairment (for review, Arhavsky, 2010), in particular short-term/episodic memory, which depends largely on hippocampal system (for review, Storandt et al., 2009; Daulatzai, 2013). This pathological and age-related memory decline is believed to be a result of reduced synaptic plasticity, including changes in the NR2 subunit composition of the NMDA receptor (for review, Wang et al., 2014). It can then evolve towards a global loss of cognitive functions defined as dementia (for review, Larson et al., 1992).

In the past, the study of infant memory has relied in models and tests used in adults and more specific amnesic patients with hippocampal damage. For this reason, the infant memory has been distinguished to declarative or explicit memory and nondeclarative or implicit memory. However, in recent years this distinction such as explicit/implicit are no longer accepted especially in relation to hippocampal function as new theories have been emerged (reviewed in Mullally and Maguire, 2014). Furthermore, there are findings that even very young infants have a more adept and flexible memory system than was previously thought and neurobiological data derived from

non-humans provide support to the new hypotheses about hippocampal development that would facilitate to interpret infant memory data from humans.

How it is Measured or Detected

In humans: The neuropsychological tests have been used for neurosensory assessment of humans including identification of altered neurobehaviours in vulnerable populations such as children (Rohlman et al., 2008). Intelligence tests, perceptual motor tests, planning tests, and logical, spatial, short term, long term, and working memory tasks can be used in neurobehavioral studies to assess learning and memory. The same test is also used to identify risks from occupational exposure to chemicals.

In laboratory animals: Current behavioural tests used for evaluating learning and memory processes in rats such as the *Morris water maze, Radial maze, Passive avoidance and Spontaneous alternation* are characterized in the KE *Decreased Neuronal Network Function*.

Cognitive function including learning and memory is an important endpoint required by the US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD 426). The methods applied to assess learning and memory have been reviewed (Markis et al., 2009) and discussed in the OECD Series on testing and assessment number 20, Guidance document for Neurotoxicity Testing (2004) . This document is considered an essential supplement to a substantial number of already existing OECD Test Guidelines relevant for neurotoxicity testing.

Regulatory Examples Using This Adverse Outcome

Impairment of learning and memory is considered a chemically-induced adverse outcome that is used for risk assessment and management purposes. Neurotoxicity testing guidelines (OECD TG 424 and 426) are implemented on a number of occasions where the neurotoxic properties of a compound have to be assessed in order to comply with relevant EU regulations. These regulations are as follows: REACH regulation (EC, No 1907/2006), Plant protection products regulation (EC, No 1107/2009), Biocidal products regulation (EC, No 528/2012), Test methods regulation (EC, No 440/2008), Classification, labelling and packaging of substances and mixtures (EC, No 1272/2008) and Maximum residue levels of pesticides in or on food and feed of plant and animal origin regulation (EC, No 396/2005).

The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD 426) both require testing of learning and memory. These DNT Guidelines have been used 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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Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Reduced, Release of BDNF	indirectly leads to	Decreased, Synaptogenesis	Moderate	Weak
Reduced, Release of BDNF	indirectly leads to	Altered, GABAergic interneurons morphology and function	Moderate	Weak
Altered, GABAergic interneurons morphology and function	directly leads to	Decreased, Synaptogenesis	Strong	Weak
Decreased, Synaptogenesis	directly leads to	Decreased, Neuronal network function in developing brain	Weak	Weak
Decreased, Neuronal network function in developing brain	directly leads to	Impairment, Learning and memory	Strong	Weak
Decreased, Thyroxine (T4) in	directly leads	Reduced, Release of BDNF	Weak	Weak

neuronal tissue	to			
Inhibition, Na+/I- symporter (NIS)	directly leads to	Decreased, Thyroidal iodide uptake	Strong	Strong
Decreased, Thyroxin (T4) in serum	directly leads to	Decreased, Thyroxine (T4) in neuronal tissue	Strong	Weak
Decreased, Thyroidal iodide uptake	directly leads to	Decreased, Thyroid hormone synthesis	Strong	Strong
Decreased, Thyroid hormone synthesis	directly leads to	Decreased, Thyroxin (T4) in serum	Strong	Strong

Reduced, Release of BDNF leads to Decreased, Synaptogenesis

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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

How Does This Key Event Relationship Work

Disruption of BDNF signaling during 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). 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 Trk-B 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 Russek, 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).

Weight of Evidence

Biological Plausibility

NMDAR activity has been linked to the signaling of the trans-synaptic neurotorophin BDNF (Neal et al., 2010). 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; Stansfiled et al., 2012; Danglot et al., 2006; Hu and Russek, 2008). 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 (Aguado et al., 2003), a critical function of the developing hippocampus and an important functional feature of the CNS.

Empirical Support for Linkage

- 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 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 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 in 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 (measured as number of puncta/neuron).

- Schjetnan 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.

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 (clioquinol) can rescue the Pb-induced impaiment 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 or 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. 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.

Finally, there are no studies directly showing that inhibition of thyroid NIS expression, functional activity or protein level are linked to a reduction of BDNF and/or synaptogenesis.

Quantitative Understanding of the Linkage

There is a lack of studies directly linking BDNF levels (gene and/or protein) with the quantitative analysis of synaptogenesis induced by decreased TH levels, and therefore no robust quantitative information can be provided. However, in the 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) direct associations between Pb-induced decrease of BDNF (protein and/or mRNA) and decrease of pre- and post-synaptic proteins are discussed, supported also by quantitative analyses of spines and dendrite morphology.

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Reduced, Release of BDNF leads to Altered, GABAergic interneurons morphology and function

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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

How Does This Key Event Relationship Work

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) as their calcium-binding protein are the ones subject to regulations by neurotrophins and BDNF specifically (Woo and Lu, 2006). These neurons do not express the BDNF protein but its functional receptor, namely the Trk-B (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 Trk-B 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 and Hu and Russek, 2008). BDNF also regulates the expression of the neuron-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 responses in developing neurons (Blaesse et al., 2009).

Weight of Evidence

Biological Plausibility

Proper function of the Central Nervous System (CNS) results from the closely regulated development and function of the neuronal cells 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 excitability and thereby the function and maturation of the neuronal networks (Voigt et al., 2001; Cherubini et al., 2011).

Many trophic factors have been plausibly implicated in the regulation of these processes but among them BDNF stands out as the prime candidate due to do 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 interneurons has demonstrated an enhanced dendritic 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 BDNF interneurons was promoted only when they were innervated by BDNF-releasing interneurons (Kohara et al., 2003). Due to these dendritic 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 exerted also by in vivo cerebellar studies, in which Trk-b 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), while a significant increase of GABAA receptor was observed in cultured hippocampus-derived neurons after treatment with BDNF (Yamada et al., 2003).

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 assumed 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 relationship, 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).

Empirical Support for Linkage

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 with 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 TrK 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 innervations 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.

- **Sawano et al., 2013** This 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). While it should be considered that BDNF brain levels were not assessed in this study, it is plausible that in these experimental settings BDNF may result downregulated, since KCC2 expression is known to be up-regulated by BDNF during development (Aguado et al., 2003; Blaesse et al., 2009), and KCC2 resulted downregulated in Sawano et al., 2013 study. Conversely, the effects of BDNF on KCC2 expression in the hippocampus encompass down-regulation in mature (adult) neurons (Rivera et al., 2002, 2004; Wake et al., 2007).

- Shiraki et al., 2012 this 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. BDNF brain levels were not assessed in this study.

- Gilbert et al., 2007 In this 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. Specifically, BDNF brain levels were not assessed in this study.

Uncertainties or Inconsistencies

Despite the well described relationship between BDNF and GABAergic interneurons morphology and function, there are several other conflicting studies. In a recent one (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. However, neither the functionality of the 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 can be activated by different neurotrophic factors and might lead to the same outcome under different conditions. This hypothesis should be further investigated, as this 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.

Furthermore, while some studies (Sawano et al., 2013; Shiraki et al., 2012) have shown a decrease of GABAergic cells 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 from gestation day 10 with either PTU at 3 or 12 ppm (0.57 or 1.97 mg/kg body weight/day) or MMI at 200 ppm (27.2 mg/kg body weight/day) 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 12 ppm PTU caused in the offspring growth retardation, lasting into the adult stage. All exposure groups showed a sustained increase of Reelin (a secreted extracellular matrix glycoprotein that plays a critical role in neuronal migration and positioning during brain development in the process regulated by TH (Alvarez-Dolado et al., 1999)) in the dentate hilus (~ 75% increase after MMI treatment) until the adult stage, in parallel with Calbindin-D-$28K^+$ cells at weaning (~ 170% increase after MMI treatment, at PND 20) and with $GAD67^+$ cells in the adult stage (~ 90% increase in 11-week-old rats treated with12 ppm PTU), indicating an increase in GABAergic interneurons.

It should be noticed that in Saegusa et al., 2010 study, increase of $GAD67^+$ cells was mainly observed in the adult stage (11-week-old rats) and analysis of GABAergic 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 studies reported a decrease of GABAergic PV^+ neurons at earlier stages, respectively on PND 15 and 21 (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. Additionally, hyperthyroidism does not always have the opposite effect, and there are 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).

Finally, apart from indirect observations on the effects of hypothyroidism (e.g., as a consequence of chemically-induced TPO inhibition), there are no studies showing that inhibition of NIS expression, functional activity or protein levels are directly linked to a reduction of BDNF levels and/or GABAergic neuron morphological or functional alterations. Further studies are needed to confirm these associations.

Quantitative Understanding of the Linkage

There is a lack of studies linking BDNF levels (gene and/or protein) and the amount of changes (morphological and function) in GABAergic interneurons and therefore no robust quantitative information can be provided.

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Altered, GABAergic interneurons morphology and function leads to Decreased, Synaptogenesis

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
Xenopus laevis	Xenopus laevis	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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

How Does This Key Event Relationship Work

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 drives 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).

Weight of Evidence

Biological Plausibility

Early in the development of the neocortex, GABAergic interneurons are implicated in the emergence of a 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., 2007), 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-]I 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 induce the end of 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-]I 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-]I 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 key event relationship.

• The 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 Mg^{2+} 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 Mg^{2+} block and allow Ca^{2+} 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 Support for Linkage

As described above, 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. 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 (≥ 25% decrease in rat cells at 4-5 DIV, ~ 70% decrease in human cells at 10 DIV) and attenuated $[Cl^-]_i$ shift in migrating cortical inhibitory precursor neurons.

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.

- Sawano et al., 2013 This study investigated the effects methimazole (MMI, a TPO inhibitor) on the developing rat hippocampus, one of the brain regions most sensitive to TH levels. MMI was administered at the concentration of 0.025% in drinking water to pregnant dams from gestational day 15 until 4 weeks postpartum. 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. This decrease correlated with a 37% reduction in the number of $GAD65^+$ cells, as well as a reduction in $GAD65^+$ processes, while GAD67 was not affected by MMI treatment. 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).

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

Uncertainties or 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 KNCC1 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.

Quantitative Understanding of the Linkage

There is a lack of studies directly linking alteration of GABAergic interneurons (morphology and function) with quantitative analyses of synaptogenesis modifications, and therefore no robust quantitative information can be provided.

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Decreased, Synaptogenesis leads to Decreased, Neuronal network function in developing brain

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Colón-Ramos has reviewed the early developmental events that take place during the process of synaptogenesis, pointing out the importance of this process in neuronal network formation and function. The experimental findings reviewed in this paper derive from knowledge acquired in the field of neuroscience using invertebrates, in particular C. elegans and Drosophila, at the same time emerging findings derived from vertebrates are also discussed (Colón-Ramos, 2009).

How Does This Key Event Relationship Work

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 may lead to decreased 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 synapse on the dendrites of the receiving cells must be intact for effective communication between neurons to be possible. Changes in the placement of cells within the network due to delays in neuronal migration, the absence of a full proliferation of dendritic arbors and spine upon which synaptic contacts are made, and the lagging of transmission of electrical impulses due to insufficient myelination will individually and cumulatively impair synaptic function. These anatomical alterations are among a host of 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 from TH modulation of the neurogenesis process in the developing hippocampus or cortex may contribute to deficits in synaptic function.

Weight of Evidence

Biological Plausibility

Neuronal network connections 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 PSD95 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, dendritic arborization, and synaptogenesis have been described in models of severe hormone deprivation. 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 the anatomical levels will very much impact the neural network function.

Empirical Support for Linkage

Most of the information on developmental hypothyroidism and altered synaptic function has been provided by study of the hippocampus. It is presumed that structural deficits at some level underlie functional deficits revealed 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 exposed animals 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).

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).

- Gilbert and Sui, 2006, Gilbert, 2011, Gilbert et al., 2016: 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.

- 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 cortical neurons in culture, T3 augments the density and growth of GABAergic neurons and accelerates the maturation of neural networks. BDNF and associated signaling molecules have been implicated in T3 modulation of network formation in vitro. In this manner, T3 modulation of activity-dependent processes represents one potential mechanism whereby thyroid hormones may refine and stabilize synaptic connectivity in the developing brain.

- Wheeler et al., 2011, 2012: In addition to the rodent data, functional magnetic resonance imaging (fMRI) data from humans also support the relationship between altered serum concentrations of TH, hippocampal structure, and synaptic function. Children of women with high TSH during pregnancy exhibit reduced hippocampal volumes (Wheeler et al., 2011) and altered patterns of hippocampal activation when engaged in memory tasks (Willoughby et al., 2013, Willoughby et al., 2014, Wheeler et al., 2015). An association between hippocampal volume and everyday memory function was observed in adolescent children born with congenital hypothyroidism (CH), despite remediation soon after birth (Wheeler et al., 2011). In a paired object recognition task, no differences were observed in task performance scores in control and CH children (Wheeler et al., 2012). However, an increased activation of hippocampus was seen in CH children engaged in this task relative to controls, and bilateral hippocampal activation was evident when in control children this was restricted to the left hemisphere. These data suggest differential synaptic activation in CH children to accomplish simple memory tasks.

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). 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 Pb^{2+} levels (less than 30 µg/dl), slow cortical potentials have been observed to be positive in children under five years old but negative in children over five years. However, age-related polarity reversal has been observed in children with higher Pb^{2+} 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.

- McCarren and Eccles, 1983: Male Sprague-Dawley rats have been exposed to Pb^{2+} 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 Pb^{2+}, whereas all groups responded to PHT with increases in primary AD duration.

Uncertainties or 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.

Quantitative Understanding of the Linkage

There are no data on the quantitative linkages between altered (hippocampal or cortical) synaptogenesis and impaired neuronal network function. Developmental window of exposure and duration of exposure can modulate response-response relationships.

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Decreased, Neuronal network function in developing brain leads to Impairment, Learning and memory

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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.

How Does This Key Event Relationship Work

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 the 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. A well-established model of memory at the synaptic levels 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.

Weight of Evidence

Biological Plausibility

Long-term potentiation (LTP) is a long-lasting increase in synaptic efficacy (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 excitatory postsynaptic currents (EPSCs) to be developed quickly and to be persistent for few weeks at least without disturbing already potentiated contacts. Once again, a substantial body of evidence has demonstrated that tight connection between LTP and diverse instances of memory exist (reviewed in Lynch, 2004).

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).

Empirical Support for Linkage

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 deficits persist into adulthood long after recovery to euthyroid status. The latter observation indicates that these alterations represent permanent changes in brain function induced from 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). Impairments in synaptic function and plasticity are observed coincident with deficits in learning tasks that require the hippocampus.

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

- Akaike, 1991; Axelstad et al., 2008: Radial arm maze deficits reported in adult offspring of rat dams treated with high doses of the TPO inhibitor, propylthiouracil (PTU), throughout gestation and lactation.

- Gilbert and Sui, 2006; Gilbert et al., 2016: Morris water maze acquisition and reversal deficits are seen in animals treated with more moderate doses of PTU and who also displayed synaptic transmission and LTP deficits in hippocampus.

- Gilbert, 2011; Gilbert et al., 2016: 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.

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:

- **Wang et al., 2016** 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 impariments: 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 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 also inhibition of NIS function) in pregnant mothers could adversely affect hippocampal neurogenesis 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.

- Oerbeck et al., 2003 This study reported visual and verbal memory deficits in congenitally hypothyroid children. All verbal memory tasks were impaired; visual memory domain gave less consistent findings.

- Wheeler et al., 2011 This study reported lower IQ scores, verbal learning deficits and everyday memory task deficits in congenitally hypothyroid children.

- Wheeler et al., 2015 This study reported paired word recognition deficits with no impairment on simple word lists. Word pairing associations, but not word list recall, are hippocampus-mediated.

- Willoughby et al., 2014 Autobiographical memory is also a memory function that is heavily dependent on the hippocampus (Willoughby et al., 2013). Congenitally hypothyroid children and children born to women with high TSH during pregnancy were weaker in recalling event and perceptual details from past naturally-occurring autobiographical events than age matched controls. They were also less accurate than controls in recalling event

and perceptual details of a staged event.

- Wheeler et al., 2012 In this study hippocampal synaptic function based on fMRI was altered while subjects engaged in a memory task. Although no differences existed between control and congenital hypothyroid (CH) adolescents in task aptitude, clear differences in the degree of hippocampus activation were seen during task engagement. fMRI revealed increased bilateral hippocampal engagement with object pair recognition task and bilateral activation, when controls revealed only left hippocampal activation.

- 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).

- D'Hooge and De Deyn, 2001 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.

- Morris et al., 1986 This study found that blocking the NMDA receptor 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.

Some epidemiological and in vivo studies have indicated associations between a lower iodide uptake, (e.g., as a consequence of exposure to perchlorate or other pollutants, such as BDE-47) and decreased cognition. For instance:

- 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 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 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.

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

Exposure to low levels of Pb^{2+} 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; 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 Pb^{2+} can impair hippocampus-mediated learning in animal models (reviewed in Toscano and Guilarte, 2005).

The majority of the studies addressing the effects of Pb^{2+} on hippocampal-associated spatial learning and memory processes have been carried out mainly in male rats (Cao et al., 2008, Gilbert et al., 2005); only a few studies have examined both sexes simultaneously (Jett et al., 1997, Xu et al., 2009).

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

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

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

exposure to Pb^{2+} occurs, in contrast to males.

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

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

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

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

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

Uncertainties or Inconsistencies

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

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

Moreover, 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 µCi $^{123}I^-$ (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.

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; Zimmermann, 2011). However, direct quantitative correlation between decreased neural network function and decreased cognition, in support to this KER, were not assessed in these reports.

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).

Quantitative Understanding of the Linkage

There is very limited information on the degree of quantitative change in neural network function required to alter cognitive behaviors. This is a result of the diversity of methods for measuring both physiology and cognitive function, which hamper cross-study analyses. This highlights the need to develop empirical data based models of this KER.

Temporal concordance of TH insufficiency and disrupted development, defined at many levels of biological organization, has repeatedly been documented by demonstrating that there are critical windows during development where permanent changes are affected. Hormone replacement studies have also demonstrated that structural alterations in brain are reduced or eliminated if T4 (and/or T3) treatment is given during the critical windows (Goodman and Gilbert., 2007; Auso et al., 2004; Lavado-Autric et al., 2003; Berbel et al., 2010; Koibuchi and Chin, 2000).

Similarly, temporal concordance of insufficient iodide uptake (as a consequence e.g., of exposure to perchlorate or other pollutants, such as BDE-47) and decreased cognitive functions has been documented by some epidemiological studies (Taylor et al., 2014; Chen et al., 2014; Roze et al. 2009) and have demonstrated that there are critical windows during gestation/development where permanent changes are affected.

Graded degrees of TH insufficiency have been also produced in dams and pups by administering varying doses of NIS inhibitor (e.g., sodium perchlorate) (van Wijk et al., 2008) or TPO inhibitors and assessing the dose-dependency of the observed effects on a variety of endpoints. In these low dose model studies, the absence of overt signs of toxicity to the dams or the nursing pups is taken as evidence of the temporal concordance of hormone insufficiency and neurodevelopmental impairment and the specificity of the observed effects on brain development to be mediated by TH insufficiency (van Wijk et al., 2008; Gilbert and Sui, 2006; Sharlin et al., 2007; Axelstad et al., 2008; Sharlin et al., 2008; Babu et al., 2011; Gilbert, 2011; Powell et al., 2012; Gilbert et al., 2013; Bastian et al., 2014; Gilbert et al., 2014; Gilbert et al., 2016).

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Decreased, Thyroxine (T4) in neuronal tissue leads to Reduced, Release of BDNF

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Birth to <1 month	Strong
Adult	Moderate

During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

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

How Does This Key Event Relationship Work

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 regulate the morphology and function of the 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 has been plausibly associated with TH (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 suggested that the thyroid insufficiency, which eventually results to lower TH levels in the brain, may lead to impairments of cognitive function by reducing the levels of BDNF mRNA or protein in the developmental brain.

Weight of Evidence

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.

Empirical Support for Linkage

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 exposing them 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 the level of TH and 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.

Compounds, such as 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and bisphenol A (BPA) have been reported to disturb TH homeostasis by inhibiting NIS-mediated iodide uptake and altering the expression of genes involved in TH synthesis (Wu Y et al., 2016).

- Byun et al., 2015 Perinatal exposure to BDE-47 (by gavaging Wistar rat dams with 0.002 and 0.2 mg/kg body weight, at gestation days 9 and 16, and at postnatal days (PND) 1, 8, and 15), elicited an decrease of *Bdnf* promoter methylation at some CpGs, which may underlie the development of cognitive deficits and perinatal hypothyroidism, as shown by analysis of 5-methylcytosine (5mC, used to measure DNA methylation) in frontal lobes from PND 41 offspring.

- Wang et al., 2011 This study assessed the effects of both PFOS and/or BDE-47 in Wistar rats, which were exposed to 3.2 and 32 mg/kg of PFOS or BDE-47 in their diet and co-exposed to a combination of each chemical (3.2 mg/kg) from gestational day 1 to PND 14. Chemicals adiministered in a combined manner synergistically downregulated BDNF expression in the cortex, in particular on PND 1.

- **Wang et al., 2016** 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 Akt (~ 51% decrease at 50 mg/kg BW/day), phospho-Akt (~ 56% decrease at 50 mg/kg BW/day), p44/42 MAPK (~ 45% decrease at 50 mg/kg BW/day) and phospho-p44/42 MAPK (~ 22% decrease at 50 mg/kg BW/day) protein levels, compared to controls, and also lower levels of phospho-CREB (~ 58% decrease at 50 mg/kg BW/day) and BDNF (~ 38% decrease at 50 mg/kg BW/day) in the hippocampus. In addition, BPA-exposed male offspring underwent memory and cognitive impariments: 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 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 phospho-ERK (~ 70% lower vs control), BDNF (~ 35% lower vs control), and phospho-CREB in F2 mice. Also, high-dose BPA (10 mg/kg) increased DNA methylation of the CREB regulated transcription coactivator 1 (Crtc1) generated in F2 mice (16–33% of CpG sites found methylated in F2 mice, whereas few of them were found methylated in F2 controls). These results suggest that BPA exposure (causing also inhibition of NIS function) in pregnant mothers could adversely affect hippocampal neurogenesis and cognitive function in future generations.

- **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, events that were also observed in hypothyroid rats.

- Chakraborty et al., 2012 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.

Additionally, in more complex models of maternal hypothyroidism 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 thyroidectomies, and T4 supplementation was performed at specific stages during gestation.

- Blanco et al., 2013 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 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. However, in this study measurement of TH levels specifically in the brain was not reported.

- Gilbert et al. 2016 A similar study showed that 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 (Bdnft, Bdnfiv, Arc, and Klf9) in adulthood and was accompanied by deficits in hippocampal-based learning.

- Abedelhaffez and Hassan, 2013 This 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 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 (TRα1) (~ 77 % decrease vs control), deiodinase type 2 (DIO2) (~ 90% decrease vs control), and BDNF mRNA expression in hippocampus (~ 75% decrease vs control).

Uncertainties or Inconsistencies

Despite the fact that many in vivo studies have shown a correlation between hypothyroidism and 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, 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.

An additional consideration is that studies in support to this KER have mainly assedd the effects of TPO inhibitors (PTU and MMI) or chemicals known to reduce iodide uptake by NIS (such as BDE-47 and BPA), while, apart from Koibuchi et al., 2001 (showing in vivo downregulation of BDFN upon co-administration with MMI and the NIS inhibitor perchlorate), there are no other studies demonstrating direct associations between NIS inhibition, brain TH levels and BDND expression or protein levels.

Quantitative Understanding of the Linkage

There are no consistent quantitative data linking TH levels in the brain and the level of BDNF expression, due to differences in study designs, dose regimes and the methods used to assess the endpoints.

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Inhibition, Na+/I- symporter (NIS) leads to Decreased, Thyroidal iodide uptake

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
Xenopus laevis laevis	Xenopus laevis laevis	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Empirical evidence comes from in vitro works using rat follicular cells, human in vitro cell models and in vivo data as well as Xenopus oocytes (Lindenthal et al., 2009).

How Does This Key Event Relationship Work

NIS is a membrane protein responsible for iodide transport 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).

Weight of Evidence

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 Iodine Transport Deficiencies (ITD) (reviewed in Spitzweg and Morris, 2010). Most of these mutations have been characterized and it is well known that they 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 still needs to be elucidated and the molecular modelling of the protein would greatly benefit these studies.

Empirical Support for Linkage

Many studies have shown inhibition of radioactive iodide uptake by using different cell models and assays where NIS function was suppressed. 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 that iodide, are also transported by NIS but Nitrate (NO_3^-), thiocyanate (SCN^-), and perchlorate (ClO_4^-) are of particular dietary and environmental importance (Jones et al., 1996; Tonacchera et al., 2004; De Groef et al., 2006). There are many studies showing the effect of inhibition of NIS on thyroidal iodide uptake:

- Cianchetta et al., 2010 For this study, the FRTL5 thyroid cell line and 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), the fluorescence of which was suppressed following NIS inhibition. Iodide uptake was quantified with the use of fluorescent changes. Perchlorate was shown to inhibit NIS by preventing iodide-induced changes in fluorescence of FRTL5 cells. Perchlorate caused a concentration-dependent inhibition of iodide uptake in the initial influx rate (IC_{50} = 1.6 μM) and in the intracellular concentration of iodide (IC_{50} = 1.1 μM). Perchlorate and iodide had the same effect in COS-7 cells expressing hNIS, meaning that they caused a concentration- and time-dependent reduction of fluorescence to those cells, but they had no effect on COS-7 cells which were not stably transfected with the hNIS. Thus, it was confirmed that the reduction of fluorescence was due to NIS anion transfer 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 (ClO-4, NO3-, SCN-). Additionally, unlabeled I- (non ^{125}I) was used to investigate the inhibition level of radioiodide uptake and to compare it with the potency of the other monoions, which are known inhibitors. The IC_{50} values for the studied compounds were the following: ClO_4^-: IC_{50} was 1.22 μM; SCN^-: IC_{50} was 18.7 μM; NO_3^-: IC_{50} was 293 μM; I^-: IC_{50} was 36.6 μM. 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 other 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 ($NaClO_4$): IC_{50} was 0.1 μM; Sodium thiocyanate (NaSCN): IC_{50} was 12 μM; Sodium nitrate ($NaNO_3$): IC_{50} was 800 μM; Sodium Tetrafluoroborate ($NaBF_4$): IC_{50} was 1.2 μM.

- Lecat-Guillet et al., 2007; 2008a A fully automated radioiodide uptake assay was used and some known NIS inhibitors were tested. A dose-dependent inhibition of iodide uptake was shown. The IC_{50} values for the studied compounds were the following: Sodium perchlorate ($NaClO_4$): IC_{50} was 1 μM; Sodium thiocyanate (NaSCN): IC_{50} was 14 μM; Sodium nitrate ($NaNO_3$): IC_{50} was 250 μM; Sodium Tetrafluoroborate ($NaBF_4$): IC_{50} was 0.75 μM. Additionally, a library of 17020 compounds was screened for the identification of new human NIS inhibitors. The identification was based on the magnitude of iodide uptake in 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. The time-dependent inhibition of the compounds that had an immediate effect in iodide uptake, suggested that they acted 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 μM. 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 μM on 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 (ITBs) was performed in Xenopus oocytes expressing NIS. The aim of this analysis was to further assess the inhibitory effect of those molecules specifically on NIS activity. 4 of those molecules 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 in the past to be a specific inhibitor of NIS (Vroye et al., 1998). It was found that dysidenin induced a rapid and reversible inhibition of about 40% of the iodide induced current in mouse NIS-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 Human studies have also used potassium perchlorate to predict the perchlorate inhibition of thyroidal iodide uptake with the use of RAIU method. Greer and co-workers tested body weight adjusted doses of potassium perchlorate and an assessment of RAIU uptake was performed on day 2 and day 14 of the treatment and one more assessment on day 15 after the treatment's termination. The no-observed-effect level (NOEL) value for inhibition of thyroidal uptake was 0.007 mg/kg-day, while the true no-effect level (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 was 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 cell line, a breast adenocarcinoma cell line, which expresses inducible

NIS by all-trans retinoic acid (ATRA) it has been shown that that inhibition of sterol regulatory element-binding proteins (SREBP) maturation by treatment with 25-hydroxycholesterol (5 µM) for 48 hr reduced ATRA (1 µM)-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 µM) in FRTL-5 cells and the rat thyroid gland (as measured in vivo by single i.p. injection of 100 µg of I^- in 500 µL of distilled water, and analysis of ^{125}I thyroid uptake), respectively, 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 µM) decreased NIS-mediated I- transport, effect that was reverted by ROS scavengers.

Uncertainties or Inconsistencies

The thyroid system is quite complex and therefore some inconsistent results have been produced by recent studies. For example, it has been observed 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 statistically underpowered.

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 µm, showed that a 60% lower value for the Km, equal to 0.59 µm. Substituting this value into the PBPK model for an average adult human significantly improved model agreement with the human RAIU data for exposures <100 µg kg^{-1} day^{-1} (Schlosser PM, 2016).

Quantitative Understanding of the Linkage

For this relationship there is not enough data to link quantitatively the inhibition of NIS with the amount of thyroidal uptake. NIS inhibition can be directly measured considering that he simultaneous transport of 2 Na^+ and 1 I^- generates a current, which could be easily measured with electrophysiological methods (Eskandari et al., 1997) or with patch clamp techniques (Van Sande et al., 2003). However, the exact stoichiometry of the molecules that are transferred is not yet known, meaning that in some cases it cannot be detected. For example, perchlorate does not cause depolarization of the cellular membrane, as it is thought to be transferred in 1 to 1 stoichiometry with the Na^+ (Van Sande et al., 2003). However, I^- uptake can also be measured in vivo, as shown in rats i.p. injected with 100 µg of I^- in 500 µL of distilled water (known to cause an inhibition of NIS- mediated I- transport), followed by analysis of radioactive ^{125}I thyroid uptake (Arriagada et al. 2015). Further studies are needed to support quantitative evaluation of this KER.

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Decreased, Thyroxin (T4) in serum leads to Decreased, Thyroxine (T4) in neuronal tissue

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	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.

How Does This Key Event Relationship Work

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 the blood. More detailed descriptions of this process can be found in reviews by Braverman 2012, Utiger 2006, and Zoeller et al., 2007.

Monocarboxylate transporter 8 (MCT8) is a specific human transporter for the T4 and T3 that allows their entry in the brain and other organs. Mutations in MCT8 (Allan-Herndon-Dudley syndrome, AHDS) lead to a severe form of X-linked truncal hypotonia, spasticity, mental retardation, and are characterised by normal to high TSH, elevated plasma T3, low T4, and decreased TH signaling in discrete brain areas (McAninch and Bianco, 2014; Anık et al., 2014; López-Espíndola et al., 2014).

Other in vivo studies have proven direct associations between levels of serum T4 and the levels of TH-dependent signalling in the brain (i.e., brain TH levels). For instance, mice characterized by single MCT8 deficiency showed low serum T4, elevated serum TSH and T3, and decreased T3-dependent gene expression in both the hypothalamus and the cortex (Stohn et al., 2016). Analogously, another study reported that MCT8 knock-out mice were characterized by high serum T3, low serum T4, and decreased forebrain TH content (Müller et al., 2014). The hippocampus seems to be one of the brain regions mostly affected by low TH levels, as shown in thyroidectomized Wistar rats, which resulted affected by hippocampal hypothyroidism (da Conceição et al., 2016). These data altogether support direct associations between levels of serum T4 and levels of TH-dependent signalling in the brain (i.e., brain TH levels).

Weight of Evidence

Biological Plausibility

The biological relationship between these two KEs is a well accepted fact within the scientific community. There is no doubt that decreased circulating T4 leads to declines in tissue contractions of T4 and T3 in a variety of tissues, including the brain.

Empirical Support for Linkage

Several studies have shown that tissue levels, including brain, of TH are proportional to serum TH levels (Oppenheimer, 1983; Morreale de Escobar et al., 1987; 1990; Calvo et al., 1992; Porterfield and Hendrich, 1992, 1993; Porterfield, 1994; Broedel et al., 2003). Empirical support for the linkage comes from pathological analyses of human brain tissues and in vivo studies on MCT8 knock-out mice and thyroidectomized rats.

Four male patients affected by AHDS from two unrelated Turkish families (age from 1.5 to 11 years) presented high plasma T3, low plasma T4 and rT3, and normal/mildly elevated TSH levels. Functional analysis of a novel missense MCT8 mutation (p.G495A) revealed lowered TH transport (from 20 to 85%) depending on the cell/tissue context. Phenotypically, patients were characterized by severe psychomotor retardation, axial hypotonia, lack of speech, diminished muscle mass, increased muscle tone, hyperreflexia, myopathic facies (Anık et al., 2014).

Another study analyzed brain sections from a 30^{th} gestational week male fetus and an 11-year-old boy in comparison with brain tissue from a 30^{th} and 28^{th} gestational week male and female fetuses, respectively, and a 10-year-old girl and a 12-year-old boy, as controls. 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 expression, abnormal calbindin-D28k content, impaired axonal maturation, and diminished biochemical differentiation of Purkinje cells. The 11-year-old boy displayed altered cerebellar structure, deficient myelination, deficient synaptophysin and parvalbumin expression, and abnormal calbindin-D28k expression (López-Espíndola et al., 2014).

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Analogously, another study reported that MCT8 knock-out mice were characterized by high serum T3, low serum T4, and decreased forebrain TH content, while TH concentrations in the liver, kidneys, and thyroid gland resulted increased (Müller et al., 2014).

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).

The hippocampus seems to be one of the brain regions mostly affected by low TH levels, as shown in thyroidectomized Wistar rats, which resulted affected by hippocampal hypothyroidism. Thyroidectomized rats were characterized by increased serum TSH, decreased T4 and T3 serum levels, and a reduced hippocampal expression of MCT8, thyroid hormone receptor alfa, deiodinase type 2, ectonucleotide pyrophosphatase/phosphodiesterase 2 and brain-derived neurotrophic factor (BDNF) genes (da Conceição et al., 2016). These data altogether support direct associations between levels of serum T4 and levels of TH-dependent signalling in the brain (i.e., brain TH levels).

Uncertainties or Inconsistencies

The ability of the developing brain to compensate for TH insufficiency is not well known. There may also be 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).

Quantitative Understanding of the Linkage

While it is a well-established fact decreased in serum TH levels result in decreased tissue TH levels, a major gap is the lack of empirical data that allow quantification of this relationship. There may also be different quantitative relationships between these two KEs depending on the compensatory ability of different developing brain regions (Sharlin et al., 2010).

- 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 and a suppression of synaptic responses in the hippocampal region of the brain of the adult offspring (Gilbert et al., 2013).

- Anık et al., 2014 Four males with AHDS from two unrelated Turkish families (age from 1.5 to 11 years) presented high plasma T3, low plasma T4 and rT3, and normal/mildly elevated TSH levels. Functional analysis of a novel missense MCT8 mutation (p.G495A) revealed lowered TH transport (from 20 to 85%) depending on the cell/tissue context.

- López-Espíndola et al., 2014 This study analyzed brain sections from a 30th gestational week male fetus and an 11-year-old boy in comparison with brain tissue from a 30th and 28th gestational week male and female fetuses,

respectively, and a 10-year-old girl and a 12-year-old boy, as controls. 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.

- Stohn et al., 2016 In this study, mice characterized by single MCT8 deficiency showed low serum T4 (~ 47% decrease vs control mice, at age P21), elevated serum TSH (~ 125% increase vs control mice, at age P21) and T3 (~ 33% increase vs control mice, at age P21), and decreased T3-dependent gene expression in both the hypothalamus (~ 57% decrease vs control mice, at age P21) and the cortex (~ 40% decrease vs control mice, at age P21), as indicated by the lower expression of the rat hairless (hr) gene, which is highly up-regulated by TH in the developing CNS.

- Müller et al., 2014 This study reported that MCT8 knock-out mice were characterized by high serum T3 (~ 100% increase at P21, and ~ 300% increase at 2.5 months of age, compared to control mice), low serum T4 (~ 62% decrease at P21, and ~ 50% decrease at 2.5 months of age, compared to control mice), and decreased TH content in the forebrain (~ 43% decrease for both T3 and T4, at 2.5 months of age, compared to control mice), while TH concentrations in the liver, kidneys, and thyroid gland resulted increased.

- da Conceição et al., 2016 Thyroidectomized Wistar rats resulted affected by hippocampal hypothyroidism. Thyroidectomized rats were characterized by increased serum TSH (~ 750% increase vs control rats), decreased T4 (~ 80% decrease vs control rats) and T3 (~ 45% decrease vs control rats) serum levels, and a reduced hippocampal expression of MCT8 (~ 83% decrease vs control rats), thyroid hormone receptor alfa (Trα1, ~ 77% decrease vs control rats), deiodinase type 2 (Dio2, ~ 90% decrease vs control rats), ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2, ~ 80% decrease vs control rats) and brain-derived neurotrophic factor (BDNF, ~ 75% decrease vs control rats) mRNAs.

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Decreased, Thyroidal iodide uptake leads to Decreased, Thyroid hormone synthesis

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

human	Homo sapiens	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

Inhibition of NIS expression and/or activity, leading to reduction of iodide uptake is known to impact TH synthesis, and this may occur also via direct or indirect inhibition of TPO, as suggested by in vivo studies in rats and using thyroid follicular rat cells. On the other hand, TPO inhibitors, such as MMI and PTU have been found to decrease NIS expression and activity in rat follicular thyroid cells vitro (Spitzweg et al., 1999). Moreover, human epidemiological studies indicate that inhibitors of iodide uptake via NIS (e.g., perchlorate and thiocyanate) have been found to raise TSH levels (Steinmaus et al., 2016b; Horton et al., 2015; Brechner et al., 2000), indicating reduced TH synthesis. Also, perchlorate was found to decrease Tg and TPO gene expression in rats, indicating reduction of TH biosynthesis (Wu F et al., 2012).

How Does This Key Event Relationship Work

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 triclosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and bisphenol A (BPA) have been reported to disturb thyroid hormone (TH) homeostasis, 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, these compounds elicit also 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).

Weight of Evidence

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 (i.e. TSH receptor and TPO inhibition) (KE down).

Empirical Support for Linkage

- **Spitzweg et al., 1999:** A 48 hr treatment of FRTL-5 cells with MMI (100 µM), PTU (100 µM), and potassium iodide (40 µM) induced ~ 50% decrease of NIS RNA 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 study showed that triclosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and bisphenol A (BPA) induced a concentration-dependent inhibition of NIS-mediated iodide uptake. Moreover, triclosan 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, triclosan and triclocarban also inhibited the activity of TPO at 166 and >300 µM, 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 (± 2.8) weeks gestation were found to positively correlate with the levels of urinary concentrations of perchlorate, nitrate and thiocyanate, with perchlorate (NIS inhibitors) having 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 whose drinking water supply was 100% perchlorate-contaminated water (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.

- Wu F et al., 2012: This study found that high dose perchlorate (520 mg/kg b.wt.) in Sprague-Dawley rats (28-day old) caused a decrease of Tg (~ 50% lower than control), and TPO (~ 45% lower than control) gene expression,

indicative of reduced TH biosynthesis, together with a decrease of FT3 (~ 50% lower than control) and FT4 levels (~ 50% lower than control), and a remarkable increase of TSH levels (125% higher than control).

Several other studies have proven that NIS inhibitors lead to a decrease of thyroidal iodide uptake (as detailed in KER1) (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 or 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 small sample sizes, short study durations, and the inclusion of mostly healthy adults (Steinmaus, 2016b).

Quantitative Understanding of the Linkage

In vitro and in vivo studies have specifically reported data supporting quantitative understanding of this KER.

- **Spitzweg et al., 1999:** this study showed that inhibition of TH synthesis (induced by TPO specific inhibitors) decreases the expression of NIS. A 48 hr treatment of FRTL-5 cells with the TPO specific inhibitors MMI (100 µM), PTU (100 µM), and potassium iodide (40 µM), induced a ~ 50% decrease of NIS RNA 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 F et al., 2012: An in vivo study found that high dose of NIS inhibitor perchlorate (520 mg/kg b.wt.) in Sprague-Dawley rats (28-day old) caused a decrease of Tg (~ 50% lower than control), and TPO (~ 45% lower than control) gene expression, indicative of reduced TH biosynthesis, together with a decrease of free T3 (~ 50% lower than control) and free T4 levels (~ 50% lower than control), and a remarkable increase of TSH levels (125% higher than control) (Wu F et al. 2012). Additional studies are needed in order to drive global conclusions about the magnitude of iodide uptake inhibition required to impact TH synthesis.

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Decreased, Thyroid hormone synthesis leads to Decreased, Thyroxin (T4) in serum

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
During brain development	Strong

Sex Applicability

Sex	Evidence
Mixed	Strong

While the 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) (Hornung et al. 2010), and some avian species (e.g., chicken) (Van Herck et al. 2013).

Despite many physiological similarities, humans and rats exhibit significantly different susceptibilities to thyroid

perturbation. For instance, dose-response data for changes in serum T3, T4, and TSH levels have been analysed from studies in humans, rats, mice, and rabbits. It was found that thyroid homeostasis in the rat appeared to be strikingly more sensitive to perchlorate (NIS inhibitor) than any of the other species. Therefore, data obtained from rat studies should be critically evaluated for their relevance to humans (Lewandowski et al., 2004).

How Does This Key Event Relationship Work

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. NIS main physiological function is to transport one iodide ion along with two sodium ions across the basolateral membrane of thyroid follicular cells. 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 and TPO are both essential for the synthesis of thyroid hormones (T3 and T4).

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

Perchlorate, thiocyanate, nitrate, and iodide, competitive inhibitors of iodide uptake, have been shown to inhibit radioactive iodide uptake by NIS (Tonacchera et al. 2004). Consequentially, these compounds seem to elicit also inhibition of TH synthesis. In particular, perchlorate blocks iodide uptake into the thyroid and decreases the production of TH (Steinmaus, 2016a).

Secretion of TH 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 THs into blood. More detailed descriptions of this process can be found in reviews by Braverman and Utiger, 2012 and Zoeller et al., 2007.

Weight of Evidence

Biological Plausibility
The biological relationship between two KEs in this KER is well accepted fact within the scientific community.

Empirical Support for Linkage
There is limited direct evidence supporting the relationship between decreased TH syntheses 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. So while empirical data is scarce, it is widely accepted fact the TPO inhibition leads to declines in serum TH levels. This is due to the fact that the sole source for circulating T4 is thyroid gland synthesis. Indeed, it has been known for decades that insufficient dietary iodine will lead to decreased serum TH concentrations due to inadequate synthesis. 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 species with known TPO inhibitors (Atterwill et al., 1990; Brown et al., 1986; Brucker-Davis, 1998; Hornung et al., 2010; Hurley et al., 1998; Kohrle, 2008).

Similarly, other studies have shown associations between NIS inhibition and decreased TH serum levels (Dong et al., 2017; Calil-Silveira et al., 2016; Tang et al., 2013; Liu et al., 2012; Pearce et al., 2012).

Temporal Evidence: there is a lack of studies that measured both TPO synthesis and TH production during development. Antonica and co-workers reported that a transient overexpression of the transcription factors NKX2-1

and PAX8 is sufficient to direct mouse embryonic stem-cell differentiation into thyroid follicular cells that organize into three-dimensional follicular structures when treated with thyrotropin. These in vitro-derived follicles showed appreciable iodide organification activity, and when grafted in vivo into athyroid mice, these follicles were able to rescue TH plasma levels, promoting subsequent symptomatic recovery (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 following development exposures (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 or 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, 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

- Fisher et al., 2013 recently published a quantitative biologically-based dose-response model (BBDR) 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. 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. Alterations in T4 homeostasis were more apparent than for T3. 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 and a suppression of synaptic responses in the hippocampal region of the brain of the adult offspring (Gilbert et al., 2013).

There are also a few other computational models that include TH 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 and has recently adapted for rat (Leonard et al., 2016). 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 THs 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.

- Steinmaus et al., 2016b Authors of this study showed that the presence of high level of perchlorate, thiocyanate, nitrate, and iodide (NIS inhibitors) in water supply could induce a decrease of total 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 TSH, as shown in 1880 pregnant women from San Diego County, California, during 2000–2003.

- Wu F et al., 2012 Authors of this study found that high dose perchlorate (520 mg/kg body weight) in Sprague-Dawley rats (28-day old) caused a decrease of Tg (~ 50% lower than control), and TPO (~ 45% lower than control) gene expression, indicative of reduced TH biosynthesis, together with a decrease of free T3 (~ 50% lower than control) and free T4 levels (~ 50% lower than control), and a remarkable increase of TSH levels (125% higher than control).

- Dong et al., 2017 Wistar rats exposed (at 0, 150, 300, and 600 mg/kg/day for 3 and 6 months) to di-(2-ethylhexyl)phthalate (DEHP), a chemical known to elicit a reduction of serum TH levels, underwent decreased of serum TH levels (FT3, FT4 in the DEHP-dosed group were lower than those in the control ($p < 0.05$); with the increase of DEHP-treated dose, rats serum TT3, TT4 and TSH level in all groups of different DEHP doses were lower than the control group at 6 M ($p < 0.05$), as well as a perturbation (i.e., a general increase) of the expression of thyrotropin releasing hormone receptor (TRHr), Deiodinases 1 (D1), thyroid stimulating hormone beta (TSHβ),

NIS, thyroid stimulating hormone receptor (TSHr), TPO, thyroid transcription factor 1 (TTF-1), and thyroglobulin (TG) genes. In particular, NIS protein, after 3 month exposure to middle (300 mg/kg/day) and high-dose (600 mg/kg/day) DEHP resulted decreased (by ~ 30% with 300 mg/kg/day, and by ~ 85% with 600 mg/kg/day), whilst after 6 months a progressive protein increase was observed. Globally these data indicate that DEHP-dependent reduction of TH serum levels occurs via a strong perturbation of the HPT axis.

- Calil-Silveira et al., 2016 Male Wistar rats treated for two months with NaI (0.05% and 0.005%) or $NaI^{+}NaClO_4$ (0.05%) (NIS inhibitors), underwent high levels of urine iodine, increased serum thyrotropin levels, slightly decreased serum TH levels, and a decreased expression of NIS, thyrotropin receptor, and TPO mRNA and protein, indicating a primary thyroid dysfunction.

- Tang et al., 2013 showed that 2,3',4,4',5-pentachlorobiphenyl (PCB118) (i.p. injected in male Wistar rats at 10, 100, or 1000 µg/kg/day, 5 days/week for 13 weeks) caused a progressive decrease of free T4, free T3 and TSH levels in serum (e.g., serum free T3, free T4 and TSH were reduced to 75%, 31% and 52%, respectively, at 1000 µg/kg/day PCB118), together with a downregulation of NIS (~ 30% at 1000 µg/kg/day PCB118) and Tg (~ 40% at 1000 µg/kg/day PCB118) mRNA expression. Moreover, PCB118 led to histopathological deterioration of the thyroid (i.e., follicular hyperplasia and expansion).

- Liu et al., 2012 This in vivo study reported that PCB153 (i.p. injected in Sprague-Dawley rats at 0, 4, 16 and 32 mg/kg/day for 5 consecutive days) caused a decrease of NIS, TPO and Tg, deiodinases, the receptors (TSHr and TRHr), together with a decrease of serum total T4, total T3, and thyrotropin releasing hormone (TRH).

- Pearce et al., 2012 A cross-sectional study conducted on 134 first-trimester pregnant women from Athens, Greece, showed an inverse correlations between the level of urinary perchlorate (NIS inhibitor) and free T3 and free T4 plasma values.

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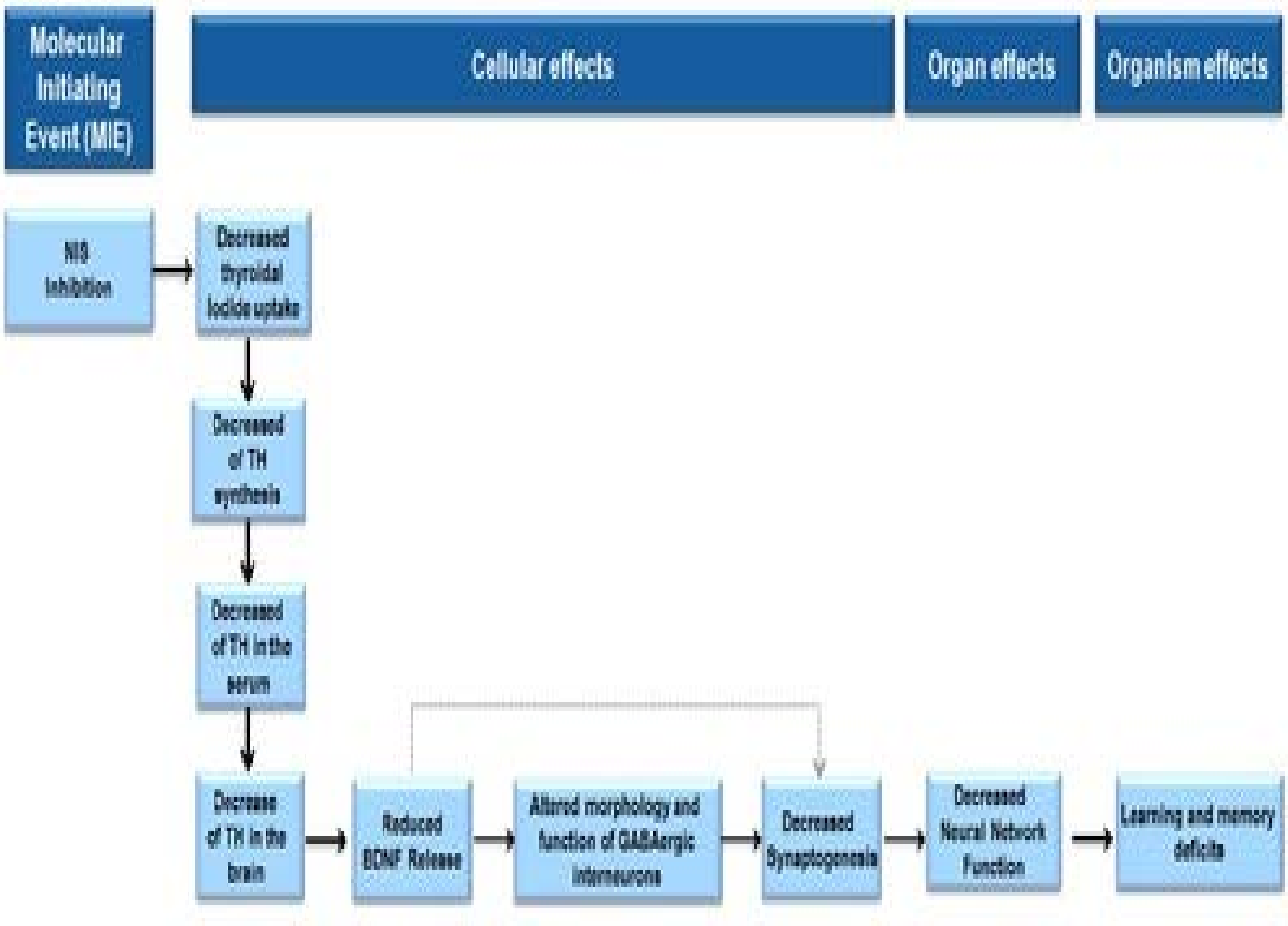
Graphical Representation



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; Pohlenz 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; Palizvan 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; Cancedda 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).

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 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; 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	Strong
Perinatal	Strong
During brain development	Strong

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Strong	NCBI
Rattus sp.	Rattus sp.	Strong	NCBI

Sex Applicability

Sex	Evidence
Male	Strong
Female	Strong

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.

Essentiality of the Key Events

The essentiality of each one of the key events in this AOP was supported by introducing a recovery period in the exposure experiments with NIS inhibitors, mainly with perchlorate. Greer et al., 2002, showed that after a recovery period of 15 days the inhibitory effect of perchlorate was eliminated, almost completely, as the measurements of iodide uptake were indistinguishable from their respective baseline values. Similar results were produced in other studies after a longer recovery period of 30 days, in which the iodide uptake as well as the serum TH levels returned to their baseline values (Siglin et al., 2000). The essential effect of NIS inhibition (MIE) to thyroidal iodide uptake (KE-downstream: decreased thyroidal iodide uptake) was also shown with the use of cells that did not endogenously express the NIS transfer protein (Cianchetta et al., 2010). In those experiments iodide was not transferred through the cellular membrane unless the cells were previously transfected with hNIS. Moreover, extensive studies on NIS protein have identified 14 different mutations and each one of them is related to Iodine Transport Deficiencies (ITD) (reviewed in Spitzweg and Morris, 2010). Several other studies have proven that NIS inhibitors lead to a decrease of thyroidal iodide uptake resulting in a reduction of TH synthesis (KE-downstream) (Jones et al., 1996; Tonacchera et al., 2004; De Groef et al., 2006; Waltz et al., 2010).

Essentiality for the association between decreased TH synthesis (KE-upstream) and decreased thyroxin (T4) in serum (KE-downstream) is proven also by studies showing the effects of NIS inhibitors on TH homeostasis and synthesis (Dong et al., 2017; Calil-Silveira et al., 2016; Tang et al., 2013; Liu et al., 2012; Pearce et al., 2012). For instance, compounds, such as triclosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and bisphenol A (BPA) have been reported to disturb TH homeostasis by inducing an inhibition of NIS-mediated iodide uptake and decreasing the expression of genes involved in TH synthesis, as shown in rat thyroid follicular FRTL-5 cells, and on the activity of TPO, using rat thyroid microsomes (Wu Y et al., 2016).

Perchlorate, thiocyanate, nitrate, and iodide, competitive inhibitors of iodide uptake, have been shown to inhibit radioactive iodide uptake by NIS (Tonacchera et al. 2004). Consequentially, these compounds also inhibit TH synthesis. In particular, perchlorate blocks iodide uptake into the thyroid gland and decreases the production of TH (Steinmaus, 2016a, 2016b).

Essentiality data for proving direct link between decreased thyroxin (T4) in serum (KE-upstream) and decreased thyroxine (T4) levels in neuronal tissue (KE-downstream), are derived from mutation studies of the monocarboxylate transporter 8 (MCT8), a specific transporter for the T4 and T3 that allows their entry in the brain and other organs. MCT8 in the CNS is expressed in the cerebral cortex, hippocampus, and amygdala, as well as in hypothalamic neuroendocrine nuclei, in tanycytes, in choroid plexus structures, and in capillary endothelial cells of the blood-brain

barrier, as highlighted by studies in mouse and human brain tissues (Mayerl et al., 2014). Mutations in MCT8 (Allan-Herndon-Dudley syndrome, AHDS) are characterised by normal to high TSH, elevated plasma T3, low T4, and decreased TH signaling in discrete brain areas (Kersseboom et al., 2013). Subjects affected by AHDS show a severe form of X-linked truncal hypotonia, spasticity, and mental retardation (McAninch and Bianco, 2014; Anık et al., 2014; López-Espíndola et al., 2014). Moreover, mice characterized by single MCT8 deficiency showed low serum T4, elevated serum TSH and T3, and decreased T3-dependent gene expression in both the hypothalamus and the cortex (Stohn et al., 2016). Analogously, another study reported that MCT8 knock-out mice were characterized by high serum T3, low serum T4, and decreased forebrain TH content (Müller et al., 2014).

Essentiality data for proving direct association between decreased T4 in neuronal tissue (KE-upstream) and reduced release of BDNF (KE-downstream) come from in vivo 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)

Essentiality data for proving direct association between decreased release of BDNF (KE-upstream) and altered GABAergic interneurons morphology and function (KE-downstream), and indirect association between decreased release of BDNF (KE-upstream) and decreased synaptogenesis (KE-downstream) come from several in vivo studies in rats, showing that prenatal exposure to TPO inhibitors (PTU or MMI, to induce hypothyroidism), decreased components of the GABAergic system (e.g., number of glutamic acid decarboxylase 65 $(GAD65)^{+}$ and number of parvalbumin $(PV)^{+}$ cells) (Sawano et al., 2013; Shiraki et al., 2012; Gilbert et al., 2007).

Moreover, a study showed that blocking BDNF with antibodies greatly reduced the formation of GABAergic inhibitory synapses (Seil and Drake-Baumann, 2000), while Yamada and colleagues found that treatment with BDNF elicited a significant increase of GABAA receptor in cultured hippocampus-derived neurons (Yamada et al., 2003). Westerholz et al., (2013), by using rat T3-deficient cultures of cortical PV^{+} interneurons, found that the number of synaptic boutons was reduced, and exogenous BDNF application abolished this effect. Also, inhibition of BDNF by K252a (a TrK antagonist) in cultures containing T3 resulted in decreased number of synaptic boutons, as in the T3-deprived cultures (Westerholz et al., 2013).

Chen and colleagues showed that GABAergic innervations of pyramidal neurons of $BDNF^{Met/Met}$ mice (characterized by a reduced activity-dependent BDNF secretion and elevated anxiety-like behaviours) are reduced at distal dendrites in hippocampal CA1 and medial prefrontal cortex, compared to wild type mice (Chen et al., 2016).

Furthermore, tyrosine receptor kinase B (TrkB, BDNF receptor) mutant mice showed reduced amounts of GABAergic markers and develop reduced numbers of GABAergic boutons and synaptic specializations (Rico et al., 2002).

In addition, Sato's 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. Importantly, E2 effects on in hippocampal slice cultures and subregional neuron cultures 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 induced by E2. Both K252a and the antibody anti-BDNF elicited a decrease of spine density and presynaptic sites (Sato et al., 2007).

Along the same line, Schjetnan and Escobar, (2012) assessed in adult rats the effects of an intrahippocampal microinfusion of BDNF, which 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. These data confirm the role of BDNF in the regulation of synaptic plasticity.

Schildt et al., (2013) performed field potential recordings in CA3 of adult heterozygous BDNF knockout (BDNF+/-) mice, and found that a decrease of NMDAR-independent mossy fiber LTP occurred in these mice. Additionally, inhibition of TrkB/BDNF signaling with K252a, or with the selective BDNF scavenger TrkB-Fc (on brain slices) both inhibited mossy fiber LTP to the same extent as observed in BDNF+/- mice.

Essentiality data for proving direct association between altered GABAergic interneurons morphology and function (KE-upstream) and decreased synaptogenesis (KE-downstream) are derived from studies on potassium chloride co-transporter 2 (KCC2), which is expressed almost exclusively in CNS neurons (Payne et al., 1996) and plays a major role in neuronal Cl^- homeostasis by maintaining a low neuronal $[Cl^-]_i$. KCC2 expression is finely regulated during brain development, and KCC2 is thought to be the regulator of GABA switch (from excitatory to inhibitory) during early neuronal development (Lee et al., 2005; Chudotvorova et al., 2005). Transcriptional repression of KCC2 in rat cortical neurons was found to delay the GABA switch, corresponding to significant changes of $[Cl^-]_i$ in GABAergic neurons (Yeo et al., 2009). Importantly, 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).

A further confirmation of KCC2 role comes from a study from Yeo and coworkers (2013), who found that bisphenol-A (BPA), a toxicant known to inhibit NIS-mediated iodide uptake (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 cortical neurons and primary human cortical neurons (Yeo et al., 2013).

Essentiality data for proving direct association between decreased synaptogenesis (KE-upstram) and decreased neuronal network function in developing brain (KE-downstream) come from studies on developmental hypothyroidism, which has been found to be associated with decreased synaptic function, particularly in the hippocampus. Structural deficits can underlie functional deficits revealed 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). Importantly, pyramidal neurons of hypothyroid animals have fewer synapses and an impoverished dendritic arbor (Rami et al., 1986, Madeira et al., 1992). Moreover, knockdown of the postsynaptic marker PSD95 arrests the functional and morphological development of glutamatergic synapses (Ehrlich et al., 2007).

Essentiality data for proving a direct association between decreased neuronal network function in developing brain (KE-upstream) and learning and memory deficits (AO) is strongly supported by several studies showing that alterations in synaptic transmission and plasticity contribute to deficits in cognitive function. 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 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).

Some hippocampal regions (i.e., 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.

Some epidemiological and in vivo studies have indicated associations between a lower iodide uptake (e.g., as a consequence of an exposure to perchlorate) and decreased cognition. For instance, Taylor and coworkers found that levels of urinary perchlorate (a NIS inhibitor) assessed in a cohort study of 21,846 women, were positively associated with a higher risk for having children with lower IQ scores at 3 years of age (Taylor et al., 2014).

van Wijk's study assessed the behavioural effects of perinatal and chronic hypothyroidism during development in offspring of hypothyroid rat dams (both dams and offspring were exposed to sodium perchlorate (NIS inhibitor) via drinking water). The Morris water maze test, used to assess cognitive performance, showed that chronic hypothyroidism negatively affected spatial memory (van Wijk et al., 2008).

Weight of Evidence Summary

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; Pohlenz 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; Palizvan 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; Cancedda 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).

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 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; 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.

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.

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