APPENDIX 1

Summary Tables - Assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements

		Defining Question	High (Strong)	Moderate	Low (Weak)
1.Support for Biological Plausibility of KERS	Direct or Indirect KER	Is there a mechanistic relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs, but the structural or functional relationship between them is not understood.
KER309: MIE => KE277 Thyroperoxidase, Inhibition - Directly Leads to - Thyroid hormone synthesis, Decreased	Direct	**Strong:** The weight of evidence supporting a direct linkage between the MIE, TPO inhibition, and the KE of decreased TH synthesis, is strong and supported by more than three decades of research in animals, including humans. Therefore, inhibition of TPO activity is widely accepted to directly impact TH synthesis.			
KER305: KE277 => KE281 Thyroid hormone synthesis, Decreased - Directly Leads to - Thyroxine (T4) in serum, Decreased	Direct	**Strong:** Thyroidal synthesis of THs is responsible for the majority of circulating THs in the blood compartment. The biological plausibility and weight of empirical evidence linking these two KEs is strong.			
KER366: MIE=>>>KE281 Thyroperoxidase, Inhibition - Indirectly Leads to - Thyroxine (T4) in serum, Decreased	Indirect	**Strong:** It is a well-accepted fact that inhibition of the only enzyme capable of synthesizing THs, TPO, results in subsequent decrease in serum TH concentrations. A large amount of evidence from clinical and animal studies clearly support the commonly accepted dogma that inhibition of TPO leads to decreased serum THs.			
KER312: KE281 => KE280 Thyroxin (T4) in serum, Decreased - Directly Leads to - Thyroxine (T4) in neuronal tissue, Decrease	Direct	**Strong** The weight of evidence for this KER is strong. It is well accepted that: the only source of neuronal tissue T4 is serum, and decreased circulating T4 leads to declines in tissue concentrations of T4 and T3 in a variety of tissues, including brain.			
KER746: KE280 => KE756 Thyroxine (T4) in neuronal tissue, Decreased - Directly Leads to - Hippocampal gene expression, Altered	Direct	**Strong** The weight of evidence is strong for TH concentrations affecting gene expression in many regions of the developing brain. Therefore, it is assumed that reductions in TH-responsive genes in the hippocampus stem from reduced availability of hormone in the brain from the serum.			
KER747: KE756 => KE757 Hippocampal gene expression, Altered - Directly Leads to Hippocampal anatomy, Altered	Direct	**Moderate** The overall weight of evidence is moderate for a direct linkage between perturbation of the expression of genes in brain (and in hippocampus specifically) and neuroanatomical abnormalities. It is widely acknowledged that the development of the structure of the hippocampus is under the control of hippocampal gene expression. However, while an extensive body of literature exists linking some genes to hippocampal structure, there is no complete compendium on the total number of genes involved, nor direct causative links between the myriad of genes and the intricate development (both timing and location) of the majority of hippocampal structure.			
KER749: KE757 => KE758 Hippocampal anatomy, Altered - Directly Leads to - Hippocampal function, Decreased	Direct	**Moderate** The weight of evidence supporting the relationship between structural abnormalities in brain induced and altered synaptic function is moderate. There is no doubt that altered structure can lead to altered function. Many examples from knock out models, genetic mutations, prenatal alcohol, nutritional deficits demonstrate a correlative link between altered structure and impaired synaptic function within the hippocampus. However, the scientific understanding of the causative and quantitative relationship between the two KEs is incomplete.			
KER748: KE758 => AO Hippocampal physiology, Decreased - Directly Leads to - Cognitive Function, Decreased	Direct	**Strong:** It is well accepted scientific dogma that the integrity of the functioning of the hippocampus is essential to spatial learning and memory in humans and a number of other mammalian species (e.g., mice, rats).			

KER1387: KE277 =>>>KE756 Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Hippocampal Gene Expression, Altered	Indirect	**Strong** The overwhelming weight of evidence for this indirect relationship is strong in humans and animals. It is entirely consistent with the known biology of how THs control brain development.
KER1388: KE277 =>>>KE757 Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Hippocampal Anatomy, Altered	Indirect	**Strong** The biological plausibility of this KER is rated as strong. The relationship is consistent with the known biology of the relationship between serum TH concentrations and brain TH concentrations and the known action of TH to modulate genes critical for developmental processes that control structural development of the brain in general, including the hippocampus. Some inconsistencies may arise when using maternal serum to predict offspring outcome on hippocampal anatomy if the kinetics of the chemical to not sufficiently reduce maternal hormones at the appropriate time, not cross the placental barrier to sufficiently disrupt fetal hormone synthesis, or are not sufficiently available to the nursing pup via the milk (European Commission, 2017)
KER1389: KE277 =>>>KE758 Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Hippocampal Physiology, Decreased	Indirect	**Strong** The biological plausibility of this KER is rated as strong. The relationship is consistent with the known biology of the relationship between serum TH concentrations, hippocampal TH concentrations, and TH control of hippocampal development and physiology.
KER403: KE277 =>>> AO Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Cognitive Function, Decreased	Indirect	**Strong** The weight of evidence for this indirect relationship is strong. Alterations in serum TH concentrations are very well correlated with adverse impacts on cognitive behaviors such as learning and memory. This includes a large amount of literature, from more than four decades of research, that links hypothyroidism and/or hypothyroxinemia with alterations in spatial cognitive function, a hippocampal dependent behavior.

2. Support for Essentiality of KEs	Defining Question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO Prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE279 (MIE): Thyroperoxidase, Inhibition	**Strong:** There is good evidence from time-course and recovery after cessation of dosing that leads to recovery of subsequent key events.			
KE277: Thyroid hormone synthesis, Decreased	**Strong:** A number of studies have demonstrated a correlation between TPO activity and decreased thyroid hormone synthesis. Thyroid gland T4 concentrations as well as serum thyroid hormones are decreased in response to thyroidectomy, and recover when in-vitro derived follicles are grafted in athyroid mice.			
KE281: Thyroxine (T4) in serum, Decreased	**Strong:** Stop/recovery experiments demonstrate recovery of serum thyroxine concentrations due to cessation of developmental exposure to TPO inhibitors. Studies in adult animals show a similar recovery after cessation of dosing.			
KE280: Thyroxine (T4) in neuronal tissue, Decreased	**Strong:** Studies have demonstrated that fetal brain T3 levels, previously decreased by maternal exposure to TPO inhibitors or thyroidectomy, recovered following maternal iv dosing with T4. In addition, upregulation of deiodinase has been shown compensate for some loss of neuronal T3.			
KE756: Hippocampal gene exprssion, Altered	**Moderate:** Most actions of TH are mediated T3 acting at the thyroid receptor to affect gene transcription. Most TH-responsive genes are activated indirectly via control of TH-dependent transcription factors so relationship to altered anatomy is complex. In addition, non-genomic targets of TH have been described.			
KE757: Hippocampal anatomy, Altered	**Moderate:** While it is widely accepted that alterations in hippocampal anatomy will affect hippocampal function. There is evidence from ex vivo studies that administration of BDNF will reverse the hippocampal dysplasia seen in Jacob/Nsfm knockout mice.			

KE758: Hippocampal physiology, Altered	**Moderate:** It is a well-accepted assertion that hippocampal synaptic integrity and plasticity are essential for spatial information processing in animals and spatial and episodic memory in humans. However, other brain regions also can influence these complex behaviors. Limited data from ex vivo studies from BDNF knockout animals that demonstrate that deficits in hippocampal synaptic transmission and plasticity can be rescued with recombinant BDNF
KE402 (AO): Cognitive Function, Decreased	**Strong:** TH replacement studies have shown recovery of cognitive function in mice born with mutant thyroid receptor alpha1, following postnatal treatment with THs. In addition, T4 therapy in congenitally hypothyroid children ameliorates a majority of cognitive deficits.

3. Empirical Support for KERs	Defining Questions	High (Strong)	Moderate	Low (Weak)
	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup > than that for KEdown? Inconsistencies?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data	Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species that don't align with hypothesized AOP
KER309: KE279(MIE) => KE277 Thyroperoxidase, Inhibition - Directly Leads to - Thyroid hormone synthesis, Decreased	**STRONG** While direct measurement of TPO inhibition following chemical exposures and subsequent synthesis suppression is rare, there is a large amount of data demonstrating reductions in the TH synthesis within the gland. Multiple studies have measured decreases in incorporation of radiolabeled precursors into THs (e.g., I-125) or TH levels in the gland following exposures to TPO inhibitors. No conflicting data exist. *Dose-response:* Dose-response data is available from a number of studies that include measured TH synthesis in response to varying doses of TPO inhibitors. Additional data is available from in vitro studies. *Temporality:* Limited data from ex vivo and in vitro measures of TPO inhibition are available that demonstrate the time course of inhibition following chemical exposures, including some data from human thyroid microsomes and ex vivo thyroid slices. This is applicable to all life stages. *Uncertainties*: Lack of in vivo studies that directly measure the time course of KE1 and KE2.			
KER305: KE277 => KE281 Thyroid hormone synthesis, Decreased - Directly Leads to - Thyroxine (T4) in serum, Decreased	**STRONG** The rate of TH production in the intact organism and thyroid explant slices can be measured for either incorporation of radiolabeled precursors (e.g., I-125) and in individual animals exposed in an experiment. Therefore, there is a fair amount of concurrent data for these endpoints. *Dose Response:* Dose-response data is available from a limited number of studies that include concurrent measures of both TH synthesis and serum TH concentrations. In addition, there are quantitative biologically-based dose-response model that predict TH synthesis changes from alterations in TPO activity. *Temporality: The temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005).* There are currently a lack of studies that measured both TPO synthesis and TH production during development. However, serum thyroid hormones recovered in athyroid mice following grafting of in-vitro derived follicles *Uncertainties*: Data for quantitative modeling of the relationship between the degree of synthesis decrease and resulting changes in circulating T4 concentrations comes from a limited number of chemcials (eg., perchlorate).			

	In addition, most of the data supporting this KER comes from inhibition of TPO, and there are a number of other processes (e.g., endocytosis, lysosomal fusion, basolateral fusion and release) that are not as well studied.
KER366: KE279 (MIE) =>>>KE281 Thyroperoxidase, Inhibition - Indirectly Leads to - Thyroxine (T4) in serum, Decreased	**STRONG** Clear evidence exists for temporal, dose-response and incidence concordance. No conflicting data exist. *Dose-Response:* There are a large number of studies that document dose-response relationships between exposure to known TPO inhibitors and decreased serum THs. There are a more limited set of studies that directly measure TPO inhibition as well as circulating TH concentrations. *Temporality*: The temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). Again a large body of literature documents the temporal nature of administered dose and serum hormone levels. *Uncertainties:* There is a need for empirical data on the quantitative relationship between the amount of TPO inhibition required to cause decreased circulating TH concentrations.
KER312: KE281 => KE280 Thyroxin (T4) in serum, Decreased - Directly Leads to - Thyroxine (T4) in neuronal tissue, Decrease	**MODERATE** A number of studies using multiple types of stressors have shown a direct relationship of serum TH to neuronal TH. *Dose-Response:* There are a limited number of studies on the correlative relationship between circulating thyroid hormone concentrations and brain tissue concentrations during fetal and early postnatal development following maternal iodine deficient diets or chemical treatments that depress serum THs. *Temporality:* The temporal nature of this KER is developmental. However, the impact of serum hormones on brain concentrations is similar across all developmental ages. Data is also available from thyroid hormone replacement studies that demonstrate recovery of fetal brain T3 and T4 levels (following low iodine diets or MMI exposure) to control levels after maternal thyroid hormone replacement or iodine supplementation. *Uncertainties:* The quantitative relationship between serum T4 and brain T4 is lacking. Other uncertainties include: compensatory mechanisms can influence local neuronal availability of T4 and these relationships have not been clarified; and there are other know steps between serum T4 and brain T, including cellular transporters and local tissue deiodinases.
KER746: KE280 => KE756 Thyroxine (T4) in neuronal tissue, Decreased - Directly Leads to - Hippocampal gene expression, Altered	**MODERATE** Only a few studies have measured neuronal TH and gene expression in the hippocampus. For these few studies, this relationship holds. *Dose-Response:* Dose-response data exists, but is limited to a small number of studies and a small number of genes. *Temporality:* The temporal nature of the relationship between serum T4 and T4 in growing neuronal tissue described is developmental (Seed et al., 2005). . The impact of brain T3 concentrations on regulation of TR regulated genes is age-dependent for a number of genes critical for normal hippocampal development. It is widely accepted that different genes are altered dependent upon the window of exposure in the fetal, neonatal or adult brain. Thyroid hormone supplementation has been shown to reverse some of the effects on gene expression *Uncertainties:* The quantitative relationship between brain T4 and gene expression is lacking. This stems from the technological challenges associated with measuring brain hormone and the sometimes subtle changes in brain gene expression induced by manipulations of the thyroid system. There is also uncertainty in our understanding of exactly which genes critical for hippocampal development are regulated by thyroid hormones.
KER747: KE756 => KE757 Hippocampal gene expression, Altered - Directly Leads to Hippocampal anatomy, Altered	**WEAK** While the number of publications in this area is extensive (e.g, Strange et al. (2014); Takei et al. (2016); and Shin et al. (2015)), there is currently an incomplete understanding of the biologicals relationships between these KEs. *Dose-Response:* Dose-response data is lacking for this KER. *Temporality:* The temporal nature of this KER is developmental. Many of the gene-anatomy relationships critical to brain development only exist during development, or exist only to a very limited extent in the adult brain. *Uncertainties:* There are no inconsistencies in this KER, but there are uncertainties. Few studies exist that report both gene expression changes and structural changes in the hippocampus in same study to provide direct causative evidence for this KER. There is no dose-response data available for this KER. Also lacking is the specific suite of genes that are altered in the hippocampus at particular developmental times that are causal to the structural defects reported. The role of potential feedback from hippocampal anatomical development and gene expression is currently unknown.
KER749: KE757 => KE758 Hippocampal anatomy, Altered - Directly Leads to - Hippocampal function, Decreased	**WEAK** Although a number of examples are evident to demonstrate direct linkages between alterations in hippocampal anatomy and disruptions in hippocampal physiology, there is not a mechanism, anatomical insult, or signature pattern of synaptic impairment that accompanies each of these treatments. *Dose-Response:* Dose-response data is lacking for this KER *Temporality:* The temporal nature of this KER is developmental. This has been demonstrated in multiple studies that have defined critical periods for manipulations that alters the structural development of the hippocampus that persists to adulthood to disrupt the synaptic physiology measured in the hippocampus. A more limited number of 'rescue' experiments have been reported.

	Uncertainties: There are no inconsistencies in this KER, but there are uncertainties. There is no data that allow for dose-response quantification between the extent of anatomical change(s) and physiological alterations. Also lacking is a mapping of types of (specific) hippocampal anatomical changes and their relationship to the different kinds of changes in hippocampal physiological function. The role of potential feedback from hippocampal physiological development on anatomical development is currently unknown.
KER748: KE758 => KER402 (AO) Hippocampal physiology, Decreased - Directly Leads to - Cognitive Function, Decreased	Strong: Empirical support for this KER is strong. The requisite of hippocampal integrity to optimal visuo-spatial context learning (i.e., episodic memory) in humans and spatial learning in rodents is well documented. In vivo recording in conscious behaving animals has demonstrated activity-dependent neural changes taking place in the hippocampus during spatial learning. Impairments in hippocampal function induced by drugs, chemicals, lesions, mutants or knock out models that cause changes in field potentials of synaptic transmission, plasticity, and hippocampal network activity, are coincident with deficits in spatial and context-based fear learning (*Temporal Evidence:* The temporal nature of this KER is developmental. This has been demonstrated in multiple studies that demonstrate critical developmental windows for disruption of the functional development of of the hippocampus and the integrity of this structure is essential for spatial ability, context learning, and fear learning. Although studies on reversibility are rare, deficits in hippocampal synaptic transmission and plasticity in slices from BDNF knockout animals can be rescued with recombinant BDNF. *Dose-Response Evidence:* Limited dose-response information is available. Studies have investigated dose-dependency of both electrophysiological and behavioral impairments in animals suffering from developmental TH insufficiency. *Uncertainties*: There are no inconsistencies in this KER, but there are some uncertainties. It is a widely held assertion that synaptic transmission and plasticity in the hippocampus underlie spatial learning (Martin et al., 2000; Gruart and Delgado-Garcia, 2007; Bramham, 2007). However, the causative relationship of which specific alterations in synaptic function are associated with specific cognitive deficits is difficult to ascertain given the many forms that learning and memory and the complexity of synaptic interactions in even the simplest brain circuits. Dose-response data useful to inform quantitative modeling of this KER is lacking.
KER1387: KE277 =>>>KE756 Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Hippocampal Gene Expression, Altered	**Moderate:** The empirical support for this KER is moderate. A number of hippocampal genes have been shown to be impacted by changes in serum TH concentrations. However, most investigations for hippocampal gene expression have employed treatments that induce severe hormone reductions induced by PTU or MMI, or by thyroidectomy. In addition, few reports have studied the same genes in the hippocampus making it difficult to compare findings. *Dose-Response Evidence:* There are a very limited number of studies that have reported on the dose-dependent nature of the correlation between serum THs and hippocampal gene expression. *Temporal Evidence:* The temporal nature of this KER is developmental. It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in altered gene expression in the developing brain, including the hippocampus. Rescue experiments for this endpoint of gene expression in hypothyroid models are very limited. *Uncertainties*: The temporal nature of this KER is developmental. It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in altered gene expression in the developing brain, including the hippocampus. Dose-response and rescue experiments for this endpoint of gene expression in hypothyroid models are limited.
KER1388: KE277 =>>>KE757 Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Hippocampal Anatomy, Altered	**Strong:** The empirical support for this KER is strong. Clinical and epidemiology studies of humans with untreated congenital hypothyroidism and severe iodine deficiency, and animal models of hyperthyroidism induces by a variety of chemical and notn-chemical stressors, all demonstrate the commonly accepted relationship between reductions in circulating levels of TH, and adverse structural alterations in brain size, including hippocampus. *Dose-Response Evidence:* There are only a limited number of animals studies available to inform the dose-dependent nature of the correlation between serum THs and changes in hippocampal anatomy. *Temporal Evidence:* The temporal nature of this KER is developmental. It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in altered hippocampal anatomy. Hypothyroidism in adult human or rodents does not produce these effects. Reversibility studies are not available for hippocampus, but are available for other brain regions in rodent models of maternal hypothyroidism *Uncertainties:* There are no inconsistencies in this KER, but there are some uncertainties. There is currently insufficient data for quantitative analysis of serum T4 and hippocampal gene expression. Dose-response data are needed to determine what degree of serum T4 decrements are required to produce alterations in the structure of the hippocampus. The exact mechanisms that underlie disruption of hippocampal anatomy cused by developmental hypothyroidism are not that recover with return to euthyroid status are distinct but have yet to be fully elucidated.
KER1389: KE277 =>>>KE758 Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Hippocampal Physiology, Decreased	Strong: Empirical support for this indirect KER is rated as strong. Empirical data from rodent studies that measure serum TH concentrations and then assess alterations in synaptic function in the hippocampus have come from a number of laboratories. This work has employed in vivo, ex vivo and in vitro preparations from developmentally exposed animals.

	Dose-Response: There are several reports on the dose-dependent nature of the correlation between serum THs and changes in hippocampal physiology albeit from a limited number of laboratories. *Temporality:* The temporal nature of this KER is developmental. It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in altered physiological function in the dentate gyrus. These effects are not observed in model of adult hypothyroidism. Rescue experiments have not been performed in developmental hypothyroid models. *Uncertainties:* There are no inconsistencies in this KER, but there are some uncertainties. It is widely accepted that changes in serum THs during development will result in alterations in hippocampal function. This has been repeatedly demonstrated. The mechanisms that underlie disruption of hippocampal neurophysiology that subserve the persistent/irreversible deficits following developmental vs those observed in adult models that recover with return to euthyroid status are distinct but have yet to be fully elucidated.
KER403: KE277 =>>> KE402 (AO) Thyroxine (T4) in Serum, Decreased INDIRECTLY leads to Cognitive Function, Decreased	**Strong:** Empirical support for this KER is rated as strong. Empirical data from studies that measure serum TH concentrations and then assess alterations in cognitive function, including hippocampal dependent behaviors, is vast. The qualitative relationship between reduced serum hormone levels and adverse cognitive outcomes is well accepted in endocrinology, as well as developmental neuroendocrinology. Indeed, the relationship between serum T4 and T3 levels and adverse neurodevelopmental outcomes (e.g., IQ loss in children) is beyond reproach. *Dose-Response:* An increasing amount of literature is now available that provides clear evidence of the 'dose-response' nature of this KER. Most research over that last 40 years has employed high doses of chemicals, or chemicals plus thyroidectomies, that results in severe depletion of circulating thyroid hormones. More recently, researchers produced graded degrees of TH insufficiency in dams and pups by administering varying doses of chemicals and have correlated them to the dose-dependency of the observed effects. *Temporality:* The temporal nature of this KER is developmental. It is a well-recognized fact that there are critical developmental windows for disruption of the serum THs that result in cognitive function. Replacement studies have demonstrated that many adverse neurobehavioral outcomes, including cognitive function, can be reduced or eliminated if T4 (and/or T3) treatment is given during the critical windows. *Uncertainties:* There are no inconsistencies in this KER, but there are some remaining uncertainties. The major remaining uncertainty is the precise relationship between the degree, timing and duration of serum TH changes that leads to cognitive deficits.