

Annex to:

[EFSA ACRONYM Panel (EFSA Panel name)] [or EFSA (European Food Safety Authority)] [or EFSA Scientific Committee], [add individual author names in the same order as it is on the first page, followed by a comma, in the format: Surname Initial(s), Surname Initial(s) and Surname Initial(s)], 20YY. [Full title, including output category]. EFSA Journal 20YY; volume(issue):NNNN, [13313686](#) pp. doi:10.2903/j.efsa.20YY.NNNN

© 20YY European Food Safety Authority. EFSA Journal published by John Wiley and Sons Ltd on behalf of European Food Safety Authority.

Draft Preamble to AOP review

EFSA is exploring ways to improve the collaboration between EFSA and OECD on the AOP Development Programme. Further cooperation regarding the AOP Development Programme where EFSA will collaborate in managing the scientific review of EFSA AOPs of relevance and of field of expertise is ongoing. The objective of the cooperation is to increase the rate of AOP scientific reviews by distributing the burden of organizing and managing those reviews and benefit AOP development and use.

EFSA has made sure that the review of this AOP has been carried out in accordance with the OECD Guidance Document (GD) No. 344 for the scientific review of Adverse Outcome Pathways (OECD, 2021) in terms of guidance principles and review content. Following these guidance principles thus guarantees that this AOP has been reviewed in line with OECD standards and can be considered for OECD endorsement and subsequent publication in the OECD working papers Series on AOP.

The principles followed are summarized here: The scientific review has been independent, and conflicts of interest avoided; Reviewers have been transparently selected as part of EFSA PPR Panel and Working Group experts; Public disclosure on AOP-Wiki of (i) the names of the reviewers and of the review manager, (ii) the collective outcome of their individual declaration of interest analysis, (iii) reviewers' comments and the responses of the AOP authors to the reviewers' comments, (iv) the summary of the scientific review, as appropriate is transparently reported; The collective scientific expertise of the Review panel covers the full scope of the AOP.

In addition, the scientific review addressed the standard set of pre-defined charge questions.

1. Scientific quality:

- o Does the AOP incorporate all appropriate scientific literature and evidence?
- o Does the scientific content of the AOP reflect current scientific knowledge

on this specific topic?

2. Weight of evidence (WoE):

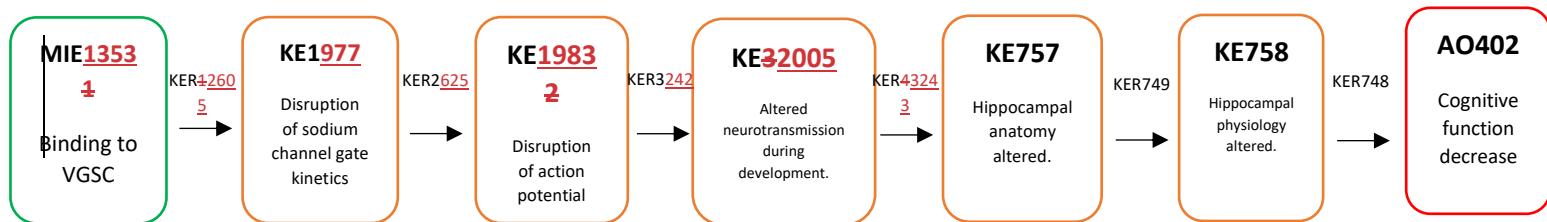
- o Is the WoE judgement/scoring well described and justified based on the evidence presented? If not, please explain.
- o Please consider WoE for each Key Event Relationship (KER) and for the AOP as a whole

Annex C_ Binding to Voltage Gate Sodium Channel (VGSC) during brain development (MiE) leading to cognitive function decrease (AOP)

Title: Binding to VGSC during development leads to cognitive function decrease

short name: Binding to VGSC during development leads to cognitive function decrease

Graphical representation



Authors

Iris Mangas, Antonio F. Hernandez, Kevin Crofton, Mary Gilbert, Martin Paparella, Anna Price, Tim Shafer, Laura Martino, Martina Panzarea, Andrea Terron

Abstract

This AOP describes one adverse outcome that may result from the binding of xenobiotics to Voltage Gate Sodium Channels (VGSC) during mammalian development. Binding to VGSC, the molecular-initiating event (MIE; KE1353), results in disruption of sodium channel gate kinetics (KE1977) and consequently to disruption of action potential generation (KE1983); this leads to a subsequent alteration in neurotransmission at all life stages, but with additional consequences when it occurs during development. Neurotransmitter release is essential for neural activity and neural activity is critical for normal brain development. Disruption of neural activity during development in many brain regions including the hippocampus can negatively impact both neuroanatomy, neurophysiology, and ultimately neurological function. Therefore, chemicals that bind with VGSCs to thwart or augment neurotransmission have the potential to cause adverse effects on the developing brain. When this occurs in the developing hippocampus, it can ultimately lead to impairments in cognitive function. Herein, we discuss the implications of developmental VGSC binding, disruption of action potential generation and neurotransmission during brain development, altered hippocampal anatomy, function, and ultimately higher cognitive processing controlled by the hippocampus. The physiology of VGSC and its essentiality for neurotransmitter release is well known across species. The hippocampus is known to be critically involved in cognitive function, including learning and memory. The adverse consequences of a chemical interference at the VGSC will depend both on severity, duration, and developmental timing, indicating that exposure could produce different effects at different developmental windows of exposure. It is important to note that this could also occur in other areas of the brain as VGSC are foundational to the structure and function of all neurons. Here we focus on the hippocampus because of its well-known ties to cognition, and downstream outcome of concern for many chemical exposures, but there is less empirical evidence and biological knowledge on the adverse consequences in other brain areas. The overall weight of evidence for this AOP is strong. Gaps in our understanding include the specific critical developmental windows and the quantitative relationship of binding to VGSC and subsequent disruption and cognitive function. Although quantitative information is limited at all levels of KERs is limited, a number of regulatory applications of this AOP for DNT assessment have been identified.

AOP Developmental Strategy

This AOP was originally started as a case study for an evidence-based AOP informed IATA for a single chemical developmental neurotoxicity hazard characterization. This case study was developed to support human health risk assessmenthazard characterization of the pyrethroid pesticidal active substance, deltamethrin, and as a proof of concept on the applicability of the data provided in the Developmental Neurotoxicity In vitro Battery. The goal of the IATA exercise was to apply mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD, 2023). A Plant Protection Products Panel EFSA Working Group of experts was established for the development of the IATA and the

outcome was published in OECD IATA Case Study Program (OECD IATA Cs n362., 2022). The IATA culminated with strong empirical evidence that deltamethrin interacts with the biological target (MIE) and may subsequently cascade through a series of measurable KEs, ultimately resulting in an adverse health outcome (AO). In this IATA Key Event Relationships (KERs) provided evidence for causality using experimental data only for deltamethrin. Although the MIE and early *event*-KEs had strong empirical support, the more downstream KEs and KERs did not. The empirical support was weak or moderate for most of the downstream KERs and when limited to evidence from only a single chemical, most KERs were not adjacent.

In order to develop a tool to apply mechanistic understanding for human health protection, a stressor agonistic AOP supported by a strong weight of evidence was developed. To do so several literature review tools were used in a step wise approach. Artificial Intelligence Tools (i.e., topic modeling) were employed to identify additional essential Key Events and to increase the scope of empirical evidence. Mapping of the literature landscape analysis culminated in identification of a new downstream KE. A chemically agnostic AOP was then developed by conducting both systematic broad and focused literature searches (i.e., searching literature using systematic search terms and, most importantly, providing a transparent description of how the literature was searched and selected) in order to collect empirical evidence in support of proposed KERs. Finally, in line with the AOP development handbook, a search was done in the AOP Wiki to find existing KEs and KERs that may be common to those in the newly developed AOP. Several downstream KEs and KERs originally described and published in AOP 42, were adopted. Finally, expert discussions of the following contributors fostered further development on this new AOP: *Iris Mangas, Antonio F. Hernandez, Kevin Crofton, Mary Gilbert, Martin Paparella, Anna Price, Tim Shafer, Laura Martino, Martina Panzarea, Andrea Terron*. Please refer also to Appendix A and Appendix C of the technical report

AOP [#442](#) Wiki

OECD status: under development

Key Events Description

MIE: KE 1353: Binding to Voltage-gated sodium channel

Short name: Binding to VGSC

Biological Context

- Level of biological organization: molecular
- Cell term: eukaryotic cell
- Organ term: cellular membrane

Key events components

- Voltage gated sodium channel activity

Other AOPs that use this KE

Key Event Overview

AOPs Including This Key Event



AOP Name	Role of event in AOP	Point of Contact	Author Status	OECD Status
Molecular events lead to epilepsy	KeyEvent	Lyle Burgoon (send email)	Open for adoption	
presynaptic neuron 1 activation to epilepsy	KeyEvent	Lyle Burgoon (send email)	Open for adoption	
Binding to VGSC during development leads to cognitive impairment	MolecularInitiatingEvent	Iris Mangas (send email)	Under development: Not open for comment. Do not cite	Under Development
Voltage-gated sodium channels and DNT	MolecularInitiatingEvent	Eliska Kuchovska (send email)	Under development: Not open for comment. Do not cite	

MIE Developmental Strategy

This MIE was originally created as part of an evidence-based AOP-informed IATA developmental neurotoxicity hazard characterization of deltamethrin (EFSA., 2021; OECD., 2022). This IATA case study was developed to support human health risk assessment of deltamethrin and as proof of concept on the applicability of the data

provided in the Developmental Neurotoxicity In vitro Battery. It was applied to provide a mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD., 2023). Using systematic searches and expert knowledge MIE descriptions and evidence was updated by an EFSA Working Group.

Taxonomic Applicability:

<u>Term</u>	<u>Scientific Term</u>	<u>Evidence</u>
mouse	<u>Mus musculus</u>	<u>High</u>
rat	<u>Rattus norvegicus</u>	<u>High</u>
human	<u>Homo sapiens</u>	<u>High</u>

Life Stages:

<u>Life stage</u>	<u>Evidence</u>
<u>All life stages</u>	<u>High</u>

Sex Applicability:

<u>Term</u>	<u>Evidence</u>
<u>Male</u>	<u>High</u>
<u>Female</u>	<u>High</u>

Life stages

All life stages

Sex applicability

Female, male

MIEs Description: Binding to voltage-gated sodium channel (VGSC)

Due to their critical role in neuronal function, sodium channels are known molecular targets of neurotoxins and neurotoxicants ([Catterall et al., 2012](#); Wakeling et al., 2012). The essentiality of sodium channels in nerve conduction comes from classic literature on tetrodotoxin (TTX). TTX is a sodium channel blocker that inhibits the firing of action potentials in neurons by binding to the voltage-gated sodium channels (VGSC/Nav) in nerve cell membranes. This action blocks the passage of sodium ions into the neuron, ions responsible for the rising phase of an action potential (AP). There is strong evidence implicating a similar TTX-like of pyrethroid insecticides on VGSC. This block of VGSC is supported by an extensive body of literature on the action of pyrethroid insecticides on mammalian sodium channels. Binding studies using radioactive pyrethroid demonstrated specific binding of the pyrethroid to rat brain VGSC α subunits (Trainer et al., 1997).

Ion channels are integral membrane proteins that are critical for neuronal function. They form "pores" in the plasma membrane that allow certain ions to travel across the membrane along their electrochemical gradient. Ion channels that open in response to a change in membrane voltage potential are called 'voltage-gated' ion channels. Channels that open in response to binding using a chemical signal or molecule are 'ligand-gated' ion channels. In neurons, ion channels of both types are essential for chemical communication between cells, i.e., synaptic transmission. Ion channels also function to maintain membrane potential and initiate AP to propagate electrical impulses. VGSC are therefore responsible for AP initiation and propagation in most excitable cells, including nerve, muscle and neuroendocrine cell types. It is important to note that functional VGSC are present in both grey and white matter in the brain. Approximately 50% of white matter oligodendrocyte precursor cells receive synaptic inputs and can produce trains of VGSC-dependent APs (Fields, 2008). VGSC are also present on microglia where they contribute to the release of major pro-inflammatory cytokines (Hossain et al., 2017).

Mammalian VGSC are composed of one α and two β subunits. Ten separate α subunits (Ogata and Ohishi, 2002) and four different β subunits (Isom, 2002) have been identified and are expressed in tissue-, region- and time- specific manners. The diverse functional roles of VGSCs depend on the numerous potential combinations of α and β subunits (Ogata and Ohishi, 2002). The type of VGSCs expressed in different cell types and regions, their sensitivity and their functional role, all contribute to the manifestation of toxicity and age-dependent sensitivity, of chemicals acting at this site.

How it is measured or detected

Interaction of compounds with VGSC can be measured directly with radioligand binding (Trainer et al 1997), while the expression and localization of VGSC on different cell types can be assessed using immunohistochemical methods. The following discussion focuses on interactions between VGSC and pyrethroids, but similar data exist for other compounds that bind to VGSC. Several other approaches provide indirect evidence of interactions of chemicals with VGSC. The published literature contains hundreds of reports identifying point mutations in VGSC that alter both the effects on the channel as well as the sensitivity to pyrethroid toxicity. Both increased and decreased modification of the insect and mammalian VGSC by pyrethroids have been demonstrated, specific action dependent on the location and type of point mutations (e.g. Vais et al., 2000; 2001). Finally, the demonstration of stereo-specific effects of the pyrethroids on binding (Soderlund 1985; Brown et al., 1988) as well as electrophysiological responses (Narahashi 1982; Narahashi 1996; Narahashi., 2000⁰²; Narahashi., 2002) also supports interaction of VGSC and pyrethroids. A model for binding of pyrethroids in insect VGSC has been developed (O'Reilly et al., 2006). Together, these observations provide strong evidence of pyrethroid binding to VGSC (for additional review, see Field et al 2017).

Biological domains of applicability

VGSCs are present in many different cell types of the ~~central~~-nervous system (CNS), including neurons, oligodendrocytes, Schwann cells (Baker, 2002; Jessen and Mirsky, 2005; Ritche, 1992; Chiu, 1991) and microglia (Jung et al., 2013; Black and Waxman reviewed in Hossain et al., 2017; Paez et al., 2009; Berret et al., 2017).

Moreover, every cell within living organisms actively maintains a low intracellular sodium concentration that is 10–12 times lower than the extracellular concentration. The cells then utilize this transmembrane sodium concentration gradient as a driving force to produce electrical signals, and if the driving force is sufficiently strong, an AP is produced. The protein family comprising VGSC (Navs) is essential for such signaling and enables cells to change their electrical status in a regenerative manner and to rapidly communicate with one another. The existence of VGSC was first predicted from studies of electrical activity in squid giant axon and later identified through molecular studies in the electric eel. Since then, these proteins have been observed in organisms ranging from bacteria to humans (Chaihne, 2018).

Sodium channels consist of highly processed α subunit, which is approximately 260 kDa, associated with auxiliary β subunits of 33–39 kDa. Sodium channels in the adult CNS and heart contain a mixture of $\beta 1$ – $\beta 4$ subunits, while sodium channels in adult skeletal muscle have only the $\beta 1$ subunit. Nine different VGSC have been identified using electrophysiological recording, biochemical purification, and cloning (Catterall, 2007; Catterall, 2012).

Nomenclature of the different sodium channel alpha (pore-forming) subunits is based on a numerical system to define subfamilies and subtypes based on similarities between the amino acid sequences of the channels. In this nomenclature system, the name of an individual channel consists of the chemical symbol of the principal permeating ion (Na) with the principal physiological regulator (voltage) indicated as a subscript (Nav). The number following the subscript indicates the gene subfamily (currently only Nav 1), and the number following the full point identifies the specific channel isoform (e.g. Nav 1.1). This last number has been assigned according to the approximate order in which each gene was identified. Splice variants of each family member are identified by lower-case letters following the numbers (e.g. Nav 1.1a). (Catterall, 2012).

In mammals, numerous neuronal VGSC are expressed in the adult and developing brain. Evidence from mutation and knockout animal models demonstrates that perturbation of VGSC function during development impairs nervous system structure and function, disrupts muscle function, pain reception, and cardiac rhythm (Chahine, 2018). VGSCs show complex regional and temporal ontogeny in mammals. Table 1, from Shafer et al., 2005 provides an overview about the alpha subunits and their developmental and tissue expression pattern. Pyrethroid interactions with Na_v1.1 (James et al., 2017), Na_v1.3 (Meacham et al., 2008; Tan and Soderlund 2009), Na_v1.6 (Tan and Soderlund, 2010), Na_v1.7 (Tan and Soderlund, 2011) and Na_v1.9 (Nutter and Cooper, 2014; Bothe et al., 2021) channels.

Table 1. Sodium channel α subunit nomenclature and effects of pyrethroids.^a

α subunit	Older names	TTX sensitivity	Tissue expression	Developmental expression	Effect of pyrethroids
Na _v 1.1	Rat I, HBSC1, GPBI, SCN1A	TTX-S	CNS, PNS, Purkinje, HP pyramidal cells, spinal motor neurons, somatic localization	Not detected in HP during development, detectable in CB Purkinje cells at PND15, detected at PND2 in SC; strong expression in motor neurons ^b	Not tested to date
Na _v 1.2	Rat II, HBSCII, HBA	TTX-S	CNS, forebrain, substantia nigra, HP mossy fibers, CB molecular layer, axonal localization	In HP, increase between GD17 and PND30; in CB granule cells on PND15 and Purkinje cells on PND2; detected at all ages in SC ^b Splice variant expressed during development ^c	Cypermethrin-induced tail currents detectable at > 30 nM in rat 1.2 (adult splice variant) co-expressed with β 1 subunits; reported insensitive to permethrin or cismethrin ^d Not tested to date
Na _v 1.3	Rat III	TTX-S	CNS and DRG	HP expression at GD17, increasing at PND2, then decreasing to barely detectable at PND30. Detected at GD17 in CB neuroepithelium, decreasing thereafter, similar in SC ^b ; developmentally regulated splice variant ^e	Not tested to date
Na _v 1.4	SkM1, μ 1	TTX-S	Skeletal muscle	Increases with age ^f	Only slightly modified by 10 μ M deltamethrin when expressed in HEK 293t cells ^g
Na _v 1.5	SkM2, H1	TTX-R	Uninnervated skeletal muscle, heart, brain	mRNA expressed in rat PND0 limbic structures and medulla; expressed in fetal and adult human brain ^h	Not tested to date
Na _v 1.6	NaCh6, PN4, Scn8a, CerrII	TTX-S	CNS, DRG (all diameter neurons), node of Ranvier–peripheral nerve	Truncated form expressed from GD12 to PND7, full-length mRNA expression is slight at GD14 and increases with age ⁱ	Not tested to date
Na _v 1.7	NaS, hNE-NA, PN1	TTX-S	DRG (all diameter neurons)	All DRG neurons at PND2, increased during development ^b	Not tested to date
Na _v 1.8	SNS, PN3, NaNG	TTX-R	DRG (small diameter neurons)	Expression beginning at GD15 with adult levels by PND7; largely in unmyelinated C-fibers ^j	Sensitive to both cismethrin and cypermethrin at thresholds of 500 nM and 30 nM, respectively ^k
Na _v 1.9	NaN, SNS2, PN5, NaT, SCN12A	TTX-R	DRG (small diameter neurons)	Expression beginning at GD17 with adult levels by PND7; largely in unmyelinated C-fibers ^j	Not tested to date
Na _v	Na _v 2.1, Na _v 2.3 Na-6, SCL11	?	Heart, uterus, skeletal muscle, astrocytes, DRG	Transient between PND2 and 15 in HP, peak expression at PND2 in CB, SC, large DRG neurons, GD17 to PND30 ^b	Not tested to date

Abbreviations: CB, cerebellum; CNS, central nervous system; DRG, dorsal root ganglion; GD, gestation day; HP, hippocampus; PND, postnatal day; PNS, peripheral nervous system; SC, spinal cord; TTX, tetrodotoxin; TTX-R, TTX resistant; TTX-S, sensitive to TTX.

^aData in the first four columns are based on information presented by Goldin et al. (2000) and Novakovic et al. (2001). ^bFelts et al. (1997). ^cSarao et al. (1991). ^dSmith and Soderlund (1998).

^eGustafson et al. (1993). ^fKallen et al. (1990). ^gWang et al. (2001). ^hDonahue et al. (2000). ⁱPlummer et al. (1997). ^jBenn et al. (2001). ^kSmith and Soderlund (2001).

β 1b and β 3 expression is high during prenatal and early postnatal period in nervous system mammals, followed by increased expression of β 1, β 2 and β 4 in the first postnatal week which then persists through adulthood. While different cell types in the brain

express different β subunits, the $\beta 1$ subunit is ubiquitously expressed with moderate heterogeneity. Its subcellular localization provides specific functionalities, e.g. high density of $\beta 1$ at the nodes of Ranvier modulates surface expression and gating of the VGSC α subunit while in the paranodal region $\beta 1$ mediates axonal-glial cell adhesion. The $\beta 2$ protein shares some similar expression pattern with $\beta 1$ and appears to provide responsiveness to inflammatory and neuropathic pain in the peripheral nervous system (PNS). In contrast $\beta 3$ mRNA and protein are expressed ubiquitously thought the developing CNS and in adult mice it is greatly reduced except for some structures like the hippocampus. This differs in human brain, where $\beta 3$ remains highly expressed throughout adulthood. The expression profile of $\beta 4$ is mostly restricted among the β subunits, and often related to neurons with spontaneous or burst firing APs. Finally, β subunits are also expressed in various glia where they may function as cell adhesion guides and cues for neurodevelopment, including coordinating neurite outgrowth, axonal fasciculation, and neuronal migration (Hull et Isom 2018). Importantly, co-expression of β subunits with the α subunit modulates the function of the α subunit and can influence the binding of various ligands to the α subunit (Tan et al., 2011). In general, embryonically expressed forms of VGSCs are replaced by expression of adult forms as neurodevelopment proceeds.

Due to this complex ontogeny of VGSCs it is currently not possible to specify which VGSCs subtypes and which developmental stages are particularly essential and thus important for this AOP.

Life Stages:

All life stages.

References

Baker MD, 2002. Electrophysiology of mammalian Schwann cells. *Progress in Biophysics and Molecular Biology*, 78(2–3), 83–103. [https://doi.org/10.1016/S0079-6107\(02\)00007-X](https://doi.org/10.1016/S0079-6107(02)00007-X)

Berret E, Barron T, Xu J, Debner E, Kim EJ and Kim JH, 2017. Oligodendroglial excitability mediated by glutamatergic inputs and Nav1.2 activation. *Nature Communications*, 8(1), 1–15. <https://doi.org/10.1038/s41467-017-00688-0>

Black JA and Waxman SG, 2012. Sodium channels and microglial function. *Experimental Neurology*, 234(2), 302–315. <https://doi.org/10.1016/j.expneurol.2011.09.030>

Bothe SN and Lampert A, 2021. The insecticide deltamethrin enhances sodium channel slow inactivation of human Nav1.9, Nav1.8 and Nav1.7. *Toxicol Appl Pharmacol.* 428,115676.<https://doi.org/10.1016/j.taap.2021.115676>

Brown GB, Gaupp JE and Olsen RW, 1988. Pyrethroid insecticides: stereospecific allosteric interaction with the batrachotoxinin-A benzoate binding site of mammalian voltage-sensitive sodium channels. *Molecular Pharmacology.* 34(1),54-9.

Catterall WA, 2012. Voltage-gated sodium channels at 60: structure, function and pathophysiology. *Journal of Physiology,* 590(11), 2577–2589. <https://doi.org/10.1113/jphysiol.2011.224204>

Catterall WA, Cestèle S, Yarov-Yarovoy V, Frank HY, Konoki K and Scheuer T, 2007. Voltage-gated ion channels and gating modifier toxins. *Toxicon,* 49(2), 124–141. doi: 10.1016/j.toxicon.2006.09.022

Chahine M (ed.), 2018. Voltage-gated Sodium Channels: Structure, Function and Channelopathies.246. Springer.

Chiu SY, 1991. Functions and distribution of voltage-gated sodium and potassium channels in mammalian Schwann cells. *Glia,* 4(6), 541–558. <https://doi.org/10.1002/glia.440040602>

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), Hernández-Jerez A, Adriaanse P, Aldrich A, Berny P, Coja T, Duquesne S, Focks A, Marinovich M, Millet M, Pelkonen O, Pieper S, Tiktak A, Topping C, Widenfalk A, Wilks M, Wolterink G, Crofton K, Hougaard Bennekou S, Paparella M and Tzoulaki I, 2021. Scientific Opinion on the development of Integrated Approaches to Testing and Assessment (IATA) case studies on developmental neurotoxicity (DNT) risk assessment. *EFSA Journal* 2021;19(6):6599, 63 pp. <https://doi.org/10.2903/j.efsa.2021.6599>

Field LM, Emry Davies TG, O'Reilly AO, Williamson MS and Wallace BA,2017. Voltage-gated sodium channels as targets for pyrethroid insecticides. *Eur Biophysics Journal.* 46(7):675-679. <https://doi.org/10.1007/s00249-016-1195-1>

Fields RD, 2008. Oligodendrocytes changing the rules: action potentials in glia and oligodendrocytes controlling action potentials. *The Neuroscientist,* 14(6), 540–543. <https://doi.org/10.1177/1073858408320294>

Hossain MM, Liu J and Richardson JR, 2017. Pyrethroid insecticides directly activate microglia through interaction with voltage-gated sodium channels. *Toxicological Sciences,* 155(1), 112–123. Oxford Academic, <https://doi.org/10.1093/toxsci/kfw187>

Hull JM, Isom LL, 2018. Voltage-gated sodium channel β subunits: The power outside the pore in brain development and disease. *Neuropharmacology*, 132:43-57. <https://doi.org/10.1016/j.neuropharm.2017.09.018>

Isom LL, 2002. β subunits: Players in neuronal hyperexcitability? *Novartis Found Symp*. 2002; 241:124-38; discussion 138-43, 226-32.

James TF, Nenov MN, Tapia CM, Lecchi M, Koshy S, Green TA and Laezza F, 2017. Consequences of acute Nav1.1 exposure to deltamethrin. *Neurotoxicology*, 60:150-160. <https://doi.org/10.1016/j.neuro.2016.12.005>

Jessen KR and Mirsky R, 2005. The origin and development of glial cells in peripheral nerves. *Nature Reviews in Neuroscience*, 6, 671–682. <https://doi.org/10.1038/nrn1746>

Jung GY, Lee JY, Rhim H, Oh TH and Yune TY, 2013. An increase in voltage-gated sodium channel current elicits microglial activation followed inflammatory responses in vitro and in vivo after spinal cord injury. *Glia*, 61(11), 1807–1821. <https://doi.org/10.1002/glia.22559>

Meacham CA, Brodfuehrer PD, Watkins JA and Shafer TJ, 2008. Developmentally regulated sodium channel subunits are differentially sensitive to alpha-cyano containing pyrethroids. *Toxicology and Applied Pharmacology*, 231(3):273-81. <https://doi.org/10.1016/j.taap.2008.04.017>

Narahashi T, 1996. Neuronal ion channels as the target sites of insecticides. *Pharmacology and Toxicology*, 79(1), 1–14.

Narahashi T, 2000. Neuroreceptors and ion channels as the basis for drug action: past, present, and future. *J Pharmacol Exp Ther*, 294, 1–26.

Narahashi T, 1982. Cellular and molecular mechanisms of action of insecticides: neurophysiological approach. *Neurobehavioral toxicology and teratology*, 4(6), 753-8.

Narahashi T. [2002](#). Nerve membrane ion channels as the target site of insecticides. *Mini Rev Med Chem*. [2002](#)-Aug;2(4):419-32.

Nutter TJ and Cooper BY, 2014. Persistent modification of Nav1.9 following chronic exposure to insecticides and pyridostigmine bromide. *Toxicology and Applied Pharmacology*, 277(3), 298-309. <https://doi.org/10.1016/j.taap.2014.04.005>

OECD, [2022](#). Case study for the integration of in vitro data in the developmental neurotoxicity hazard identification and characterisation using deltamethrin as a prototype chemical; Series on Testing and Assessment No. 362. Available at: [https://one.oecd.org/document/env/cbc/mono\(2022\)24/en/pdf](https://one.oecd.org/document/env/cbc/mono(2022)24/en/pdf)

OECD, 2023. Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)

Ogata N and Ohishi Y, 2002. Molecular diversity of structure and function of the voltage-gated Na^+ channels. Japanese Journal of Pharmacology, 88(4), 365–377. <https://doi.org/10.1254/jjp.88.365>

O'Reilly AO, Khambay BP, Williamson MS, Field LM, Wallace BA and Davies TG, 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. Biochemical Journal.396(2):255-63. <https://doi.org/10.1042/BJ20051925>

Paez PM, Fulton D, Colwell CS and Campagnoni AT, 2009. Voltage-operated Ca^{2+} and Na^+ channels in the oligodendrocyte lineage. Journal of Neuroscience Research, 87(15), 3259–3266. <https://doi.org/10.1002/jnr.21938>

Ritchie JM, 1992. Voltage-gated ion channels in Schwann cells and glia. Trends in Neurosciences, 15(9), 345–351. [https://doi.org/10.1016/0166-2236\(92\)90052-A](https://doi.org/10.1016/0166-2236(92)90052-A)

Soderlund DM, 1985. Pyrethroid-receptor interactions: stereospecific binding and effects on sodium channels in mouse brain preparations. Neurotoxicology. 1985 Summer;6(2):35-46.

Tan J, Choi JS and Soderlund DM, 2011. Coexpression with Auxiliary β Subunits Modulates the Action of Tefluthrin on Rat $\text{Na}(\text{v})1.6$ and $\text{Na}(\text{v})1.3$ Sodium Channels. Pesticide Biochemistry and Physiology, 101(3), 256-264. <https://doi.org/10.1016/j.pestbp.2011.10.003>

Tan J, Soderlund DM, 2011. Actions of Tefluthrin on Rat $\text{Na}(\text{v})1.7$ Voltage-Gated Sodium Channels Expressed in Xenopus Oocytes. Pestic Biochem Physiol, 101(1):21-26. <https://doi.org/10.1016/j.pestbp.2011.06.001>

[Tan J, Soderlund DM, 2009. Human and rat Nav1.3 voltage-gated sodium channels differ in inactivation properties and sensitivity to the pyrethroid insecticide tefluthrin. Neurotoxicology, 30\(1\), 81-9. doi: 10.1016/j.neuro.2008.10.008.](https://doi.org/10.1016/j.neuro.2008.10.008)

Tan J, Soderlund DM, 2010. Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat $\text{Na}(\text{v})1.6$ sodium channels. Toxicology and Applied Pharmacology, 247(3), 229-37. <https://doi.org/10.1016/j.taap.2010.07.001>

~~Tan J, Söderlund DM, 2009. Human and rat Nav1.3 voltage-gated sodium channels differ in inactivation properties and sensitivity to the pyrethroid insecticide tefluthrin. Neurotoxicology, 30(1), 81-9. doi: 10.1016/j.neuro.2008.10.008.~~

Trainer VL, McPhee JC, Boutelet-Bochan H, Baker C, Scheuer T, Babin D, and Catterall WA, 1997. High affinity binding of pyrethroids to the α subunit of brain sodium channels. Molecular Pharmacology, 51(4), 651–657. doi: <https://doi.org/10.1124/mol.51.4.651>

Vais H, Atkinson S, Eldursi N, Devonshire AL, Williamson MS and Usherwood PN, 2000. A single amino acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides. FEBS Lett, 470(2):135-8. [https://doi.org/10.1016/S0014-5793\(00\)01305-3](https://doi.org/10.1016/S0014-5793(00)01305-3)

Vais H, Williamson MS, Devonshire AL, and Usherwood PN, 2001. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. Pest Management Science, 57(10), 877-88. <https://doi.org/10.1002/ps.392>

Wakeling EN, Neal AP and Atchison WD, 2012. Pyrethroids and their effects on ion channels. Pesticides—Advances in Chemical and Botanical Pesticides. Rijeka, Croatia: InTech, pp. 39–66. Available at: <http://dx.doi.org/10.5772/50330>.

Abbreviations

AO	Adverse outcome
AOP	Adverse outcome pathway
AOP-Wiki	Collaborative Adverse Outcome Pathway Wiki
AP	Action Potential
CNS	Central nervous system

DNT	Developmental neurotoxicity
EFSA	European Food Safety Agency
IATA	Integrated Approach to Testing and Assessment
KEs	Key Events
KERs	Key event relationships
kDa	Kilo dalton
KNP	Key neurodevelopmental processes
MIE	Molecular initiating event
OECD	Organisation for Economic Co-operation and Development
PNS	Peripheral nervous system
TTX	Tetrodotoxin
WG	Working Group
WoE	Weight of evidence
VGSC/Nav	Voltage Gate Sodium Channel

KE1:KE1977: Disruption of sodium channel gating kinetics

Short name

Altered kinetics of sodium channel

Biological Context

- Level of Biological Organization: Molecular
- Cell term: eukar^otic cells

- Organ term: nervous system

Key events components

Key Event Components			
	Process	Object	Action
KE 584 Inhibition of sodium channel	sodium channel inhibitor activity	sodium channel protein type 1 subunit alpha	decreased
	signaling	sodium channel protein type 1 subunit alpha	Decreased
KE 598 modulation, sodium channel	sodium channel inhibitor activity	sodium channel protein type 1 subunit alpha	decreased
KE599 prolonged, Depolarization of neuronal membrane	membrane depolarization		increased
KE 763 hyperpolarisation, neuron	membrane hyperpolarization		increased

- Other sodium channel related KEs are blank in this section

Process: channel kinetics

Object: VGSCs

Action: changed

Other AOPs that use this KE

- <https://aopwiki.org/aops/489>

KE Developmental Strategy

This KE was originally created as part of an evidence-based AOP informed IATA for deltamethrin for developmental neurotoxicity hazard characterization. The IATA case study was developed to support human health risk assessment of deltamethrin pesticide active substance and as a proof-of-concept on the applicability of the data provided in the Developmental Neurotoxicity In vitro Battery to apply mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD., 2023). Using systematic searches and expert knowledge the initial MiE was updated by an EFSA Working Group.

Life stages

All life stages

Sex applicability

Female, male

Taxonomic Applicability:

<u>Term</u>	<u>Scientific Term</u>	<u>Evidence</u>
<u>mouse</u>	<u>Mus musculus</u>	<u>High</u>
<u>rat</u>	<u>Rattus norvegicus</u>	<u>High</u>
<u>human</u>	<u>Homo sapiens</u>	<u>High</u>

Life Stages:

<u>Life stage</u>	<u>Evidence</u>
<u>All life Stages</u>	<u>High</u>

Sex Applicability:

<u>Term</u>	<u>Evidence</u>
<u>Male</u>	<u>High</u>
<u>Female</u>	<u>High</u>

KE1 Description: Disruption of sodium channel gating kinetics

Action potentials (AP) are a temporary shift (from negative to positive) in the neuron's membrane potential caused by ions flowing in and out of the neuron. During the resting state, before an action potential occurs, voltage-gated sodium and potassium channels are predominantly closed. These gated channels only open once when an action potential has been triggered. They are called 'voltage-gated' because they are open and close depending on the voltage difference across the cell membrane. VGSCs have two gates (gate m and gate h), while the potassium channel only has one (gate n). Gate m (the activation gate) is normally closed and opens when the cell membrane potential starts to get more positive (depolarizes). Gate h (the deactivation gate) is normally open, and swings shut when the cell membrane potential gets too positive. Gate n is normally closed, but slowly opens when the cell is depolarised (very positive). VGSCs exist in one of three states: Deactivated/closed (closed), activated (open) and inactivated (closed) – at rest, channels are (Figure 1) .

Modifications of the sodium channel gating have been studied using voltage and patch clamp experiments in different models (Ruigt et al., 1987). Prolongation of the sodium current is mainly due to the reduced rate of closure of a fraction of the sodium channel population and is characterized by a 'tail current'. In neuroblastoma cell preparations, chemical stressors including deltamethrin and other type II pyrethroids, induce a slow tail current with a relatively long time constant. The rate at which sodium channels close during the pyrethroid-induced slow tail current depends not only on pyrethroid structure, but also on the duration of exposure, temperature and membrane potential (Ruigt et al., 1987; Narahashi., 2002; Soderlund., 2002).

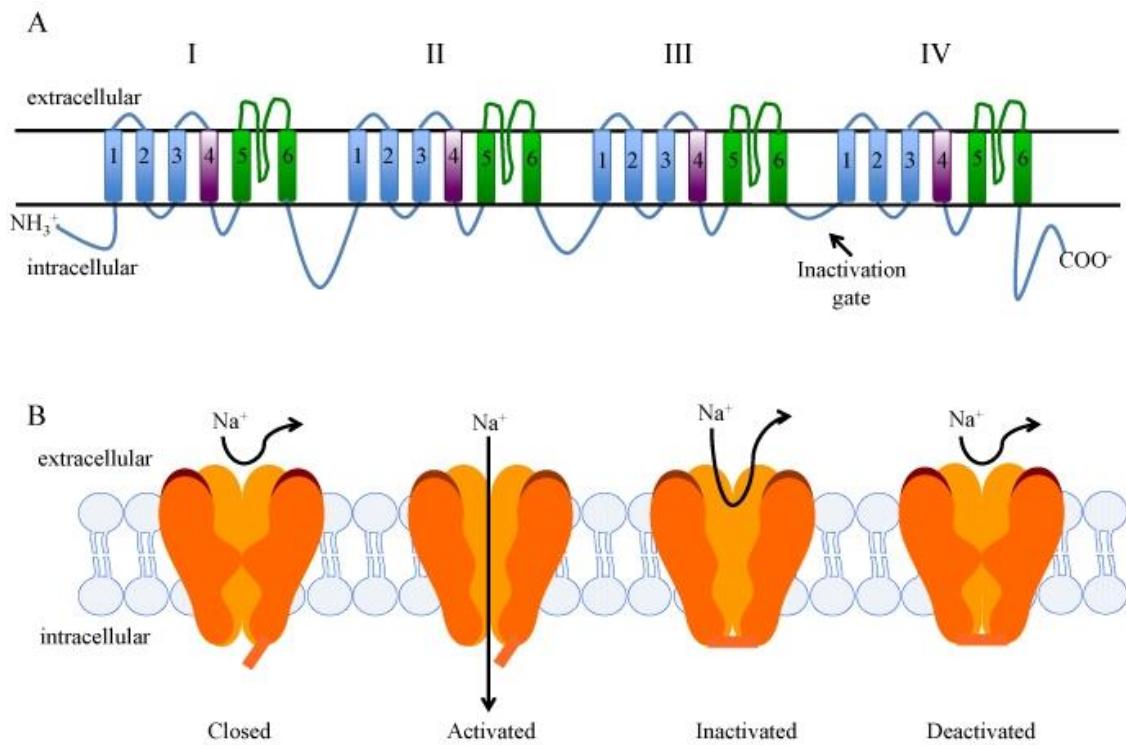


Figure 1. The three existing states of the VGSCs: Deactivated (closed), activated (open) and inactivated (closed). [Figure extracted from](#) Wakeling et al., 2012)

How it is measured or detected

Typically, VGSC function is measured using electrophysiological approaches, as only these have sufficient temporal resolution to evaluate channel function. Voltage-clamp techniques typically use two microelectrodes, allowing control of the membrane potential ('clamping') and recording of transmembrane currents that result from ion channel opening and closing (Guan et al., 2013). Pharmacological approaches and modifications of the ionic composition of the solution are used to isolate currents passing through VGSC from other types of current in the neuron.

In the patch-clamp technique, a highly sensitive version of the voltage-clamp technique, a single glass microelectrode is attached to a neuron to form a tight seal between the glass pipette tip and the cell membrane. In this case, a single electrode controls voltage and passes current (Mollemann, 2003). Typically, the current measured is the sum of currents flowing through the entire population of channels in this patch of membrane, the 'whole cell' patch configuration (Hamill et al., 1981). Some configurations of patch clamp technique can measure current flowing through a single ion channel. Most studies utilizing this technique involve in vitro or ex vivo measurements.

Other approaches can be used to indirectly measure VGSC function, including radiotracer flux, fluorescent approaches, and calcium imaging. While these approaches can provide useful information in many cases, they are not direct, nor do they have sufficient resolution to fully describe VGSC function ([Molleman A, 2003](#)).

Biological domains of applicability

Ion channels are essential for the initiation and propagation of APs in excitable cells in both vertebrate and invertebrate species. In neurons, ion channels are essential for chemical communication between cells, or synaptic transmission. Ion channels also function to maintain membrane potential and initiate and propagate electrical impulses. VGSC are a target of natural and synthetic chemicals and disruption of the gate kinetics has been characterized in insects and mammalian cells (Soderlund et al., 2002).

For more details [and references](#) see [also](#) the description in MIE: KE 1353 Binding to Voltage Gated Sodium Channel

References

Guan B, Chen X and Zhang H, 2013. Two-electrode voltage clamp. Methods in Molecular Biology, 998, 79–89. doi: 10.1007/978-1-62703-351-0_6

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Pflugers. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Archiv: European journal of physiology, 391(2), 85–100. <https://doi.org/10.1007/BF00656997>

Molleman A, 2003. Patch Clamping: An Introductory Guide to Patch Clamp Electrophysiology. John Wiley and Sons. DOI:10.1002/0470856521

Narahashi T. (2002). Nerve membrane ion channels as the target site of insecticides. Mini reviews in medicinal chemistry, 2(4), 419–432. <https://doi.org/10.2174/1389557023405927>

OECD,2023 Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)

Ruigt GS, Neyt HC, Van der Zalm JM, and Van den Bercken J,1987. Increase of sodium current after pyrethroid insecticides in mouse neuroblastoma cells. Brain research, 437(2), 309–322. [https://doi.org/10.1016/0006-8993\(87\)91645-3](https://doi.org/10.1016/0006-8993(87)91645-3)

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo V J, Sargent D, Stevens JT and Weiner ML, 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology*, 171(1), 3–59. [https://doi.org/10.1016/s0300-483x\(01\)00569-8](https://doi.org/10.1016/s0300-483x(01)00569-8)

Glossary [and/or] Abbreviations

APs	Action Potentials
AOP	Adverse outcome pathway
DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
EFSA	European Food Safety Agency
IATA	Integrated Approach to Testing and Assessment
KEs	Key Events
MIE	Molecular initiating event
VGSCs	Voltage Gate Sodium Channels

KE2: KE1983: Disruption in action potential generation

Short name: Disruption in action potential

Biological Context

- Level of Biological Organization: Cellular
- **Cell term**

~~Further information on Event Components and Biological Context may be viewed on the attached pdf. The biological context describes the location/biological environment in which the event takes place. For molecular/cellular events this would include the cellular context (if known), organ context, and species/life stage/sex for which the event~~

~~is relevant. For tissue/organ events cellular context is not applicable. For individual/population events, the organ context is not applicable.~~

- **Organ term**
- ~~Further information on Event Components and Biological Context may be viewed on the attached pdf. The biological context describes the location/biological environment in which the event takes place. For molecular/cellular events this would include the cellular context (if known), organ context, and species/life stage/sex for which the event is relevant. For tissue/organ events cellular context is not applicable. For individual/population events, the organ context is not applicable.~~

Key event components

- Biological process: action potential
- Object: disruption
- Action: disruption

Other AOPs that use this KE

- **none**

KE Developmental Strategy

As described for MiE and KE1, this KE was originally created as part of an evidence based AOP informed IATA for deltamethrin developmental neurotoxicity hazard characterization. This IATA case study was developed to support human health risk assessment of deltamethrin pesticide active substance and as a prove of concept on the applicability of the data provided in the Developmental Neurotoxicity In vitro Battery (DNT IVB-) to apply mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD., 2023). Using systematic searches and expert knowledge the initial MiE description and evidence was updated by an EFSA Working Group.

Life stages

~~All life stages~~

Sex applicability

~~Female, male~~

Taxonomic Applicability:

<u>Term</u>	<u>Scientific Term</u>	<u>Evidence</u>
mouse	<u>Mus musculus</u>	<u>High</u>
rat	<u>Rattus norvegicus</u>	<u>High</u>
human	<u>Homo sapiens</u>	<u>High</u>

Life Stages:

<u>Life stage</u>	<u>Evidence</u>
<u>All life Stages</u>	<u>High</u>

Sex Applicability:

<u>Term</u>	<u>Evidence</u>
<u>Male</u>	<u>High</u>
<u>Female</u>	<u>High</u>

Key event description

Generation of action potentials (APs)

The action potential is a transient depolarization and repolarization of the membrane that occurs in electrically excitable cells (neurons, cardiac cells, muscle). Due to unequal distribution of charged ions between the inside and outside of the cell, a voltage difference (potential) exists between the intracellular and extracellular sides of the cell membrane. At "resting" levels of activity, this membrane potential is about -70 millivolts. Typically, sodium ions level are much higher outside of the cell while potassium ions are higher inside of the cell. The action potential is initiated by a depolarizing stimulus that

results in the opening of voltage-gated sodium channels in the membrane. Once a few sodium channels open, this triggers local depolarization opening other nearby VGSC and induction of a rapid depolarization to positive potentials (e.g. +20 mV) of the membrane. This rapid depolarization is due to influx of positively charged sodium ions into the cell through the VGSC. Sodium channels rapidly “inactivate”, closing even if the membrane remains depolarized, limiting the amount of sodium entering the cell and stopping additional depolarization. The VGSC induced depolarization of the membrane causes voltage-gated potassium channels to open, which results in positively charged potassium ions exiting the cell to repolarize the membrane to its resting potential. In myelinated neurons, myelin introduces insulation around the axon that allows depolarization to spread further down the axon with great efficiency. Sodium channel expression is higher around specialized “nodes” in the myelin sheath, nodes of Ranvier. The high concentration of VGSCs at these nodes increase the probability that a sufficient number of VGSC will open on depolarization to reach the threshold for firing an action potential. In this manner the sequestering of VGSC at the node allows the electrical impulse to quickly jump from node to node along the length of the axon, increasing the speed of propagation of the action potential.

The process described above is highly conserved across electrically excitable cells and is described in a generic manner here. Due to the diversity of different neuron types and expression of different combinations of ion channels across those neuron types, differences in the shape and temporal patterns of APs are observed across different cells in the nervous system. The basic components of the action potential as described above are well conserved in neurons from invertebrates to vertebrates including mammals and humans.

For an easy-to-read summary description including figures of action potential generation and propagation, see e.g.

- <https://openbooks.lib.msu.edu/neuroscience/chapter/action-potentials/>
- <https://pressbooks.umn.edu/sensationandperception/chapter/action-potentials/>

How it is measured or detected

The action potential is a cycle of membrane depolarization, hyperpolarization and return to the resting value. It is measured most directly using electrophysiological approaches, which allow measurements to be made on a time scale that is consistent with the speed at which these events occur (milliseconds). Other approaches allow for more indirect assessments of APs using optical and other measures. Typically, these optical approaches do not have the temporal resolution of electrophysiological measurements.

Electrophysiological Techniques ~~for~~ Measurements of Action Potentials

There are a wide variety of electrophysiological techniques that allow for action potential measurement. At their core, all of them allow the recording of changes in either membrane potential or currents flowing across the membrane, and all ~~are capable of doing~~ can do so with high temporal resolution (milliseconds) necessary to record APs. Different configurations each have inherent advantages and disadvantages and the selection of the appropriate technique depends on the specific questions to be addressed by an experiment. All these approaches make use of one or more electrodes to measure the electrical responses (changes in membrane voltage or current) in a cell or group of cells. The electrodes can be of various sizes and shapes, and may be placed inside the cell (intracellular recordings), on the cell (patch clamp recordings), or adjacent to the cell (extracellular recordings). [Please, see Khadria, 2022 and Ogden, 1994 for further details.](#)

In patch recording, in contrast to evaluating specific channel activity as described in KE1 for VGSC activation, to evaluate AP, the current clamp configuration is commonly employed. Also distinct from KE1, pharmacological manipulations are not applied so that all channel types can contribute to the AP response - AP requires both sodium and potassium ion flow. The action potential is reflected in a rapid fluctuation in voltage.

In the intracellular recording, sharp glass microelectrodes are inserted directly into the intracellular space of the neuron and membrane voltage is measured. Membrane voltage increases dramatically once a threshold depolarization is reached and an action potential is reflected in a short duration steep increase in voltage followed by a rapid fall. Compared to patch clamp, sharp electrode intracellular recording is more difficult to perform, but allows recordings to be obtained for much longer periods of time. They measure the synaptic signals of cells with a high signal-to-noise ratio.

Rather than on or in the cell, extracellular recordings show changes in the activity of several cells surrounding a microelectrode. Alterations to the position and size of this electrode will change the nature of the measurement. These are referred to as unit recordings where APs are characterized by high frequency of activity on msec timescale. Often, action potential signals from multiple cells are recorded on the same electrode and one cell distinguished from the other using a process called spike sorting. Spike sorting uses computer algorithms to analyze the waveforms of the electrical activity and separate them based on their temporal profile, amplitude and other characteristics.

Microelectrode arrays (MEAs) are a form of extracellular recording, consisting of chips that contain multiple electrodes, typically arranged in a small grid. Rather than recording from a single electrode, action potential signals can be recorded from multiple electrodes simultaneously. The number of electrodes ranges from tens to thousands depending on the spatial resolution of the array and type of data required by the experiment. Different types of arrays can be used for a wide variety of *in vitro* and *in vivo* applications. MEA

recordings provide multiple parameters of electrical activity, with firing rate and bursting rates as the most common to characterize APs.

Local field potentials are also extracellular recordings, but measure the synchronized electrical potential of a group of cells whose source may be difficult to determine. The signals from these cells will overlap and the recording will be a sum of all of the electrical activity. Commonly used in laminated structures with known anatomical inputs, stimulating electrodes are placed on the input presynaptic axonal field and electrical responses induced after a short synaptic delay represent neurotransmission from pre to postsynaptic neurons.

Optical measurements of action potential

A variety of optical techniques are used as indirect measurement of the action potential. These have the advantage of being higher throughput than electrophysiological approaches, but the disadvantage of having a slower temporal resolution. They are highly correlated with action potential generation, but are subject to some confounders and do not possess the temporal resolution of electrophysiological approaches. These include the use of Na^+ or Ca^{++} sensitive dyes that fluoresce in response to the binding of one of these ions. Single APs are not detected, but changes in bulk ion concentration over a finite period of time primarily reflect the firing rate of the cells. Another optical technique uses dyes that are sensitive to changes in voltage or fluorescence resonance energy transfer (FRET) using specialized fluorophores that respond based on changes in membrane potential. Large changes in FRET fluorescence are indicative of changes in electrical activity. Under the correct conditions, FRET fluorescence can reflect changes in action potential generation.

For additional information on these techniques see Khadria, 2022 and Ogden, 1994.

Biological domains of applicability

Action potentials or nerve impulses are rapid and transient electrical activity that are propagated in the membrane of excitable such as neurons and muscle cells. The same principal mechanism exists in all cells and therefore is independent of sex. Action potentials are present from fetal stages on in vertebrates and they are also present in invertebrates.

References

Khadria A,2022. "Tools to Measure Membrane Potential of Neurons". Biomedical Journal ,45 (5),749–62. <https://doi.org/10.1016/j.bj.2022.05.007>.

Ogden D(ed.) ,1994. Microelectrode techniques. The Plymouth Workshop Handbook. Cambridge, The Company of Biologists Ltd, 448pp.<http://plymsea.ac.uk/id/eprint/7954/>

OECD, 2023 Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)

Glossary [and/or] Abbreviations

APs	Action Potentials
AOP	Adverse outcome pathway
DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
EFSA	European Food Safety Agency
FRET	Fluorescence resonance energy transfer
IATA	Integrated Approach to Testing and Assessment
KE	Key Event
MEAs	Microelectrode arrays
MIE	Molecular initiating event
VGSCs	Voltage Gate Sodium Channels

KE3: KE 2005: Altered neurotransmission during development

Short name: Altered neurotransmission

Biological Context

- Level of Biological Organization: Cellular
- **Cell term** neuron
- **Organ term** brain

Key event components

- Biological process: Neurotransmission
- Object: synapse
- Action: disruption

Other AOPs that use this KE

- **none**

KE Developmental Strategy

As with other KEs in this AOP, KE3 was originally created as part of an evidence-based AOP-informed IATA developmental neurotoxicity hazard characterization of deltamethrin. This IATA case study was developed to support human health risk assessment of deltamethrin and as proof of concept on the applicability of the data provided in the Developmental Neurotoxicity In vitro Battery. It was applied to provide a mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD., 2023). Using systematic searches and expert knowledge the initial MiE and KE descriptions and evidence were updated by an EFSA Working Group.

Taxonomic Applicability:

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

Life Stages:

Life stage	Evidence
During brain development. Could occur in all life stages. This KE as described is applicable to developmental period because the consequences	High

<u>Life stage</u>	<u>Evidence</u>
<u>of altered neurotransmission can be different when occurring during development or in adult life stage</u>	

Sex Applicability:

<u>Term</u>	<u>Evidence</u>
<u>Male</u>	<u>High</u>
<u>Female</u>	<u>High</u>

Life stages

~~Could occur in all life stages. This KE as described is applicable to developmental period because the consequences of altered neurotransmission can be different when occurring during development or in adult life stage.~~

Sex applicability

~~Female, male~~

Key event description

The arrival of the nerve impulse at the presynaptic terminal of the nerve's axon stimulates the release of neurotransmitter into the synaptic cleft. The neuron is a secretory cell and the secretory product, the neurotransmitter, is released to span the distance between neurons, the chemical synapse.

Neurotransmitters synthesized by the neuron are stored in the presynaptic element, inside the pre-synaptic vesicles. Release of neurotransmitter can be described by probabilistic principles. The probability of neurotransmitter release is very low under normal "resting" conditions but increases dramatically upon depolarization of the axonal nerve terminal by an action potential (AP). AP depolarization of the presynaptic site of the axon terminal causes voltage gated Ca^{2+} channels to open. Calcium ions entering the cell initiate a signalling cascade that causes small membrane-bound vesicles, called pre-synaptic vesicles, containing neurotransmitter molecules to fuse with the presynaptic

membrane. Neurotransmitters are then released, diffuse across the space between the presynaptic and postsynaptic neurons, the synaptic cleft, and bind to their appropriate receptors on the postsynaptic membrane. Signalling is then terminated by three different mechanisms: diffusion of the neurotransmitter out of the synaptic cleft, degradation of neurotransmitter by specific enzymes (e.g. acetylcholinesterase), or reuptake of the neurotransmitter by glia cells and the presynaptic neuron.

In principle different neurons excrete excitatory or inhibitory neurotransmitters, inducing in the postsynaptic membrane either a depolarisation or hyperpolarisation, respectively. In consequence these actions either trigger or impede the generation of a new postsynaptic depolarization. Neurons integrate the various excitatory and inhibitory signals they receive from the large number of synapses with their presynaptic network, resulting in a net signalling event in their postsynaptic targets.

Disruption of neurotransmission during development can result in permanent changes in nervous system function.

How it is measured or detected

Neurotransmission can be measured by a wide variety of different approaches. The same technologies described in KE2 for AP generation can be used to measure neurotransmission by applying different protocols. These include patch clamp, intracellular and extracellular recordings, microelectrode array (MEA) recordings. KE 2005 can be measured using many methodologies that examine neural connectivity (i.e., neurotransmission), including the *in vitro* NNF assay. A standardized NNF test system to assess the potential impact of chemical exposure on neural network formation and function has been developed using rodent cortical neurons (Frank et al., 2017). Depending on the type of recording used, electrophysiological techniques capture presynaptic events such as the action potential arriving at the terminal that induces a release of neurotransmitter, or post-synaptic events, such as post-synaptic excitatory or inhibitory responses. These responses are induced by binding of neurotransmitter to postsynaptic membrane receptors. These electrical signals are the consequence of neurotransmitter diffusion from the pre- to the postsynaptic element, binding of neurotransmitter molecules to postsynaptic receptors and induction of the postsynaptic current.

Biochemical assessment of neurotransmission (i.e. *in vivo* microdialysis or *in vitro* measurement of neurotransmitters released into the media) are also common and well described in the literature.

PleaseFor further details, see Khadria, (2022) and Ogden, (1994). for further details.

Patch Clamp

Intracellular recordings measure neurotransmission following stimulation of presynaptic neurons via excitatory and inhibitory currents by electrodes positioned inside the postsynaptic neuron. Similar currents can be assessed using patch clamp techniques.

In extracellular field potential recordings stimulating electrodes placed on the input presynaptic axonal field induce after a short synaptic delay a response in the postsynaptic cells reflecting neurotransmission.

In microelectrode arrays, neurotransmission is reflected by parameters including synchronized network activity, correlation of neuronal activity across multiple electrodes, and activity evoked by direct stimulation.

As described above for KE2, Action Potential Generation, optical approaches can also be used to measure neurotransmission, such as the use of pH sensitive dyes that are incorporated into the pre-synaptic vesicles, dyes that change fluorescent properties once released into the synaptic cleft due to a difference in pH between the vesicle compartment and the synaptic cleft.

Biological domains of applicability

The process of neurotransmission is generally similar in structure/function across most taxa with nervous systems (Libenskind et al. 2017; Roschchina 2010). This includes studies using both *in vitro* and *in vivo* methods. In vertebrates, synaptic transmission usually travels in one direction. The process of synaptic transmission has been well-studied and is described in many standard neurophysiology textbooks.

Components of the synapse include (a) a presynaptic module, in which calcium signals are transduced into chemical secretions (known as excitation–secretion coupling); (b) a postsynaptic module (postsynaptic density), which comprises the proteins that support the specialized postsynaptic membrane and the signalling that goes on there; and (c) a module that determines the specific wiring diagram of neurons during development (axonogenesis). Connections between neurons can be, in this way, mapped by acquiring and analyzing electron microscopic wiring diagrams. —For the module “c”, during development, and after injury, axons must grow and find their correct synaptic targets. Electrical activity in neurons and neural networks can stabilize structural connections at the synapse, while loss of activity leads to elimination of synapses. The proteins

responsible for this targeting include secreted and membrane-bound signals and receptors that have not been as well studied in an evolutionary framework as for the other modules.

Despite its apparent specialization for neuronal signalling, the excitation–secretion system in neurons comprises many ancient gene families. However, like the transduction module, these gene families are often used differently in the various animal lineages. The proteins involved in docking and in recycling are, for the most part, conserved across eukaryotes (Liebeskind et al. 2017).

Several neurotransmitters have been found not only in animals, but also in plants and microorganisms. Thus, the presence of neurotransmitter compounds has been shown in organisms lacking a nervous system and even in unicellular organisms. Today, we have evidence that neurotransmitters, which participate in synaptic neurotransmission, are multifunctional substances participating in developmental processes of microorganisms, plants, and animals (Roschchina 2010). In the brain, many neurotransmitters also act as trophic factors in early brain development.

The neurotransmission wiring code, including excitation–secretion coupling, postsynaptic density and axonogenesis is present across multiple taxa and represents a fundamental brain developmental process.

References

Khadria A,2022. "Tools to Measure Membrane Potential of Neurons". Biomedical Journal ,45 (5),749–62. <https://doi.org/10.1016/j.bj.2022.05.007>.

Liebeskind BJ, Hofmann HA, Hillis DM and Zakon HH ,2017. Evolution of animal neural systems, Annual review of ecology, evolution, and systematics, 48, 377-398, Annual Reviews, <https://doi.org/10.1146/annurev-ecolsys-110316-023048>.

Ogden D(ed.) ,1994. Microelectrode techniques. The Plymouth Workshop Handbook. Cambridge, The Company of Biologists Ltd, 448pp.<http://plymsea.ac.uk/id/eprint/7954/>

Roshchina VV,2010. Evolutionary considerations of neurotransmitters in microbial, plant, and animal cells. In: Lyte, M., Freestone, P. (eds) Microbial Endocrinology, pp. 17-52. Springer, New York, NY. https://doi.org/10.1007/978-1-4419-5576-0_2

Liebeskind BJ, Hofmann HA, Hillis DM and Zakon HH ,2017. Evolution of animal neural systems, Annual review of ecology, evolution, and systematics, 48, 377-398, Annual Reviews, <https://doi.org/10.1146/annurev-ecolsys-110316-023048>.

~~Reshchina VV, 2010. Evolutionary considerations of neurotransmitters in microbial, plant, and animal cells. In: Lyte, M., Freestone, P. (eds) Microbial Endocrinology, pp. 17-52. Springer, New York, NY. https://doi.org/10.1007/978-1-4419-5576-0_2~~

Glossary [and/or] Abbreviation

AOP	Adverse outcome pathway
AP	Action potential
DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
ITA	Integrated Approach to Testing and Assessment
KEs	Key Events
MEA	Microelectrode array
MIE	Molecular initiating event

KE4: KE757: Hippocampal anatomy altered

KE 757 has been extracted from the AOP wiki on October 10th. Changes proposed by EFSA WG were discussed and agreed with the KE 757 developers in various WG meetings.

Short name

Hippocampal anatomy altered

Biological Context

- Level of Biological Organization: **Tissue**
- Organ term: **brain**

Key event components

- Biological process: brain development
- Object: hippocampal formation

- Action: morphological change

Other AOPs that use this KE:

AOP Name	Role of event in AOP	Point of Contact	Author Status	OECD Status
Thyroperoxidase (TPO) Inhibition and Altered Neurodevelopment	KE	Kevin Crofton	Open for citation & comment	WPHA/WNT Endorsed
Nuclear receptor induced Thyroid hormone (TH) Catabolism and Developmental Hearing Loss	KE	Katie Paul Friedman	Open for adoption	Under Development
Sodium Iodide Symporter (NIS)and Cognitive Dysfunction	KE	Mary Gilbert	Under Development: Contributions and Comments Welcome	
Transthyretin interference	KE	Kristie Sullivan	Under Development: Contributions and Comments Welcome	Under Development
Thyroid receptor (TR) Antagonism and DNT	KE	Kevin Crofton	Under development: Not open for comment. Do not cite	Under Development

Taxonomic Applicability:

Term	Scientific Term	Evidence
mouse	<i>Mus musculus</i>	High
rat	<i>Rattus norvegicus</i>	High
human	<i>Homo sapiens</i>	High

Life Stages:

Life stage	Evidence
During brain development	High

Sex Applicability:

Term	Evidence
Male	High
Female	High

KE Developmental Strategy

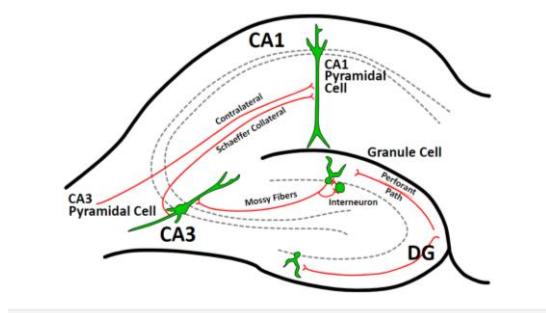
KEs in this AOP, KE4 was originally created as part of an evidence-based AOP-informed IATA developmental neurotoxicity hazard characterization of deltamethrin. This IATA case study was developed to support human health risk assessment of deltamethrin and as proof of concept on the applicability of the data provided in the Developmental Neurotoxicity In vitro Battery. It was applied to provide a mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD., 2023). Using systematic searches and expert knowledge the initial MiE and KE descriptions and evidence were updated by an EFSA Working Group.

Key event description

The hippocampus is a **major** brain region located in the medial temporal lobe in humans and other mammals (West, 1990). Developmentally it is derived from neuronal and glial cells in the neural tube and differentiates in the proencephalon and telencephalon. The hippocampus is a cortical structure, but only contains 3-layers, distinct from the 6-layered neocortical structures. For this reason, it is known as archicortex or paleocortex meaning old cortex. In humans, at the macro level, the structure is identified as early as fetal week 13 and continues to mature until 2 to 3 years of age (Kier et al., 1997), with continuing slow growth thereafter until adult ages (Utsunomiya et al., 1999). In rodents, the hippocampus begins to form in mid-gestation, with the CA fields forming in advance of the dentate gyrus (Altman and Bayer, 1990a; 1990b). **Generally speaking, the** **The** primary structural and functional development of the hippocampus occurs in the third trimester of pregnancy in humans, whereas in rodents, much of the maturation of the CA fields and almost all dentate gyrus occurs in the first 2-3 postnatal weeks.

The structure of the hippocampus has been divided into regions that include CA1 through CA4 and the dentate gyrus. The principal cell bodies of the CA field are pyramidal neurons, those of the dentate gyrus are granule cells.

The major input pathway to the hippocampus is from the layer 2 neurons of the entorhinal cortex to the dentate gyrus via the perforant path forming the first connection of the trisynaptic loop of the hippocampal circuit (Figure 2). Direct afferents from the dentate gyrus (mossy fibers) then synapse on CA3 pyramidal cells which in turn send their axons (Schaeffer Collaterals) to CA1 neurons to complete the trisynaptic circuit. Information from the CA fields then passes through the subiculum entering the fiber pathways of the alveus, fimbria, and fornix and is routed to other areas of the brain (Amaral and Lavenex, 2006). Through the interconnectivity within the hippocampus and its connections to amygdala, septum and cortex, the hippocampus plays a pivotal role in several learning and memory processes, including spatial behaviors. The primary input pathway to the CA regions of the hippocampus is from the septum by way of the fornix and direct input from the amygdala. Reciprocal outputs from the hippocampus back to these regions and beyond also exist.



Trisynaptic circuit of hippocampal formation. The dentate gyrus (DG) receives input from entorhinal cortex neurons whose axon travel as a fibre tract called the perforant path. These axons terminate on granule cell dendrites of the DG. The 2nd synapse of the circuit is mossy fibre axons of DG granule cells that synapse on pyramidal cells of the CA3 subfield of the hippocampus. The 3rd synapse of the circuit is the axons of CA3 neurons, the Schaffer Collateral that synapse on dendrites of CA1 pyramidal cells. Axons from the contralateral CA3 field cross the midline to also innervate the CA1 neurons.

Figure 2. [Trisynaptic circuit of hippocampal formation. For further details see Amaral and Lavenex, 2006.](#)

At the cellular level, the components of the mammalian hippocampus undergo typical stages of neurodevelopment. With each developmental time window, distinct patterns of gene transcription and protein expression appear, corresponding to cell proliferation, differentiation, migration, synapse formation, and terminal neuronal/glial maturation, culminating in the structural formation of a neuronal network (Mody et al., 2001; Laeremans et al., 2013). The principal neurons of the CA fields develop in advance of the principal cells of the dentate gyrus and the genes and proteins controlling the distinct phases are expressed at different stages in these two sub-regions (Altman and Bayer, 1990 a, b; Laeremans et al., 2013). In the rodent brain, almost all neurons show extensive growth and differentiation on axons and dendrites during the first postnatal week. These cellular changes are marked by rapid protein expression specific for different neuronal and glial subtypes including cytoskeletal proteins (e.g. cofilin, actins, tubulins etc.), production of cell adhesion molecules, and extracellular matrix formation which are critical structural elements of a neuronal network.

As neurons mature, they extend dendritic processes that lengthen and branch, the ends of which broaden to form a spine head. Dendritic spines form the postsynaptic structural component of most excitatory synapses in the mammalian brain, including hippocampus. The spine head has a greater potential for connectivity and synapse formation (Dailey and Smith, 1996; Fiala et al., 1998; Hardy, LR and Redmond, 2008, Pfeiffer et al., 2018). The postsynaptic density-95 (PSD-95) is one of the key proteins involved in dendritic spine maturation, clustering of synaptic signalling proteins, and ultimately mediating synaptic transmission. It also plays a critical role in regulating dendrite outgrowth and branching and formation dendritic spines.

As the hippocampus matures during the postnatal period hippocampal circuits become more active and exhibit increased activity-dependent plasticity. Many genes and proteins are upregulated during this phase of development, especially molecules involved in the axon guidance (e.g. BDNF/CREB) (Hinkemeyer et al., 2003; Shen and Cowan., 2010), dendritic spine formation (e.g. Neuroligin, Ephrins) and synaptogenesis. Increased expression of vesicle associated proteins (e.g., SNAP-25), synaptic vesicle proteins (e.g., synaptophysin, synapsin I) and proteins involved in sodium and calcium-mediated transmitter release occurs during this period. These changes are accompanied by a parallel increase in neurotrophins and neurotransmitters, receptors and ion channels (Sudhof, 2018; Zhong et al., 2020; Rizo and Rosenmund, 2008). Therefore, any alterations in the expression of these proteins (Figure 3) may result in changes of synapse formation, followed by alteration of neuronal networks within the hippocampus.

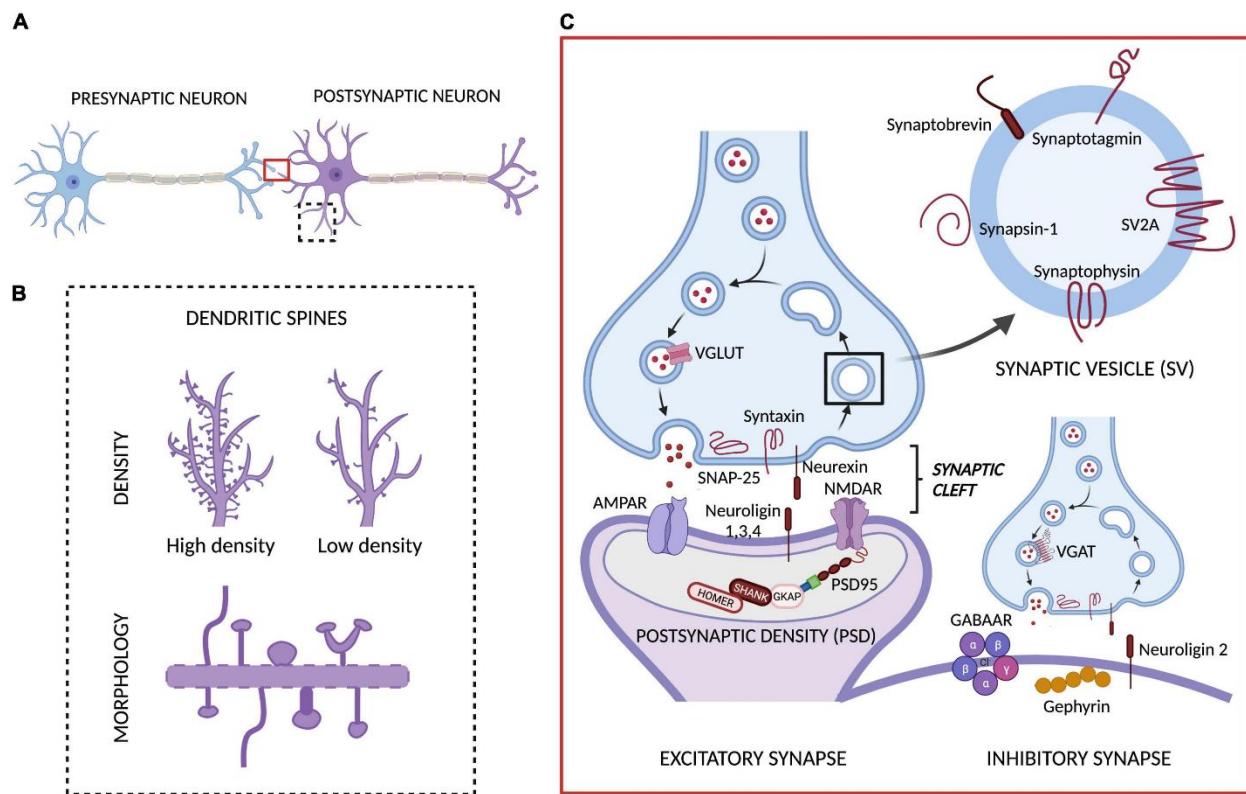


Figure 3. The main structural components of the synapse: (A) a presynaptic and a postsynaptic neuron, separated by a synaptic cleft; (B) the dendritic spines and (C) the proteins involved in synaptic formation and transmission, including synaptic vesicle (SV), presynaptic and postsynaptic proteins present in excitatory and inhibitory synapse. Adapted from Serrano et al., 2022.

The dendritic spine represents the primary site of synaptic activity at the postsynaptic site. A variety of proteins present in the presynaptic terminal and the postsynaptic dendritic spine, are expressed at different times during synaptogenesis, perturbation of which can negatively impact synaptic formation and structure at the macro- and ultra-structural level.

The use of genetically modified mouse models has been widely applied to delineate a host of different proteins involved in the structural development of the hippocampus (Joo et al., 2020). With this approach, changes in neuronal morphology, synapse and network formation in the hippocampus *is are* contrasted in animals lacking this protein *vs* 'wild type' mice where the protein has been maintained. These comparisons have adopted a variety of techniques, several described below. In KE4, differential expression of proteins identified in these model systems is taken as evidence of altered structure.

However, it is essential to consider the timing of events during development, when their detection is optimal (Hevner, 2007; Garman et al., 2001; Zgraggen et al., 2012). Some macrolevel structural changes may be transient yet still significantly impact downstream

events. In the case of knockout models, it is also important to recognize that in most cases, the protein has been removed for the entire lifespan of the animal, in the brain and elsewhere in the body, a scenario distinct from a chemical perturbation.

How It Is Measured or Detected

Data in support of this key event have been collected using a wide variety of standard biochemical, molecular, cellular, histological and anatomical methods (e.g., morphometrics, protein quantification using different types of cellular staining, immunohistochemistry, and imaging procedures) at different stages of hippocampus development. Many of methods applied are routine neurohistopathology procedures [similar to like](#) those recommended in EPA and OECD developmental neurotoxicity guidelines (US EPA, 1998; OCED, 2007). The quantification of cell body and neurite proteins can be carried out by performing immunocytochemistry and automated high content imaging analyses in *in vitro* and *in vivo* preparations (Harrill and Mundy, 2011, Meng et al., 2002), Pistollato et al., 2020). Subtle cytoarchitectural features depend on more specialized birth dating procedures and staining techniques. At the micro- and ultra-structural levels, changes in neuronal and glial morphology, alterations in synapse structure, dendritic spine formation (size, shape, number, distribution of head/neck ratios in hippocampal cultures or *in vivo* studies) and dendritic morphology (branching points, length etc.) can be assessed in Golgi-Cox impregnated neurons (Bongmbaa et al., 2011), two-photon microscopy (Ehrlich et al., 2007), transmission electron (TEM) and [fluorescent](#) [fluorescent](#) microscopy (Runge et al., 2020; Pchitskaya et al., 2020)

Two-photon time-lapse images can be used to visualise dendrites in GFP-transfected neurons, whereas Golgi Stain is used to measure both dendrites and dendritic spines. A combination of Golgi-Cox and immunofluorescence using confocal microscopy has also been suggested for the visualisation of dendrites in brain slices derived either from rodents or non-human primates (Levine et al., 2013).

Fluorescent markers, such as [Dil](#) [I](#) (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) permits not only the visualisation of detailed dendritic arborizations and spines in cell culture and tissue sections but is also compatible with the quantitative analysis of dendritic spine number (Cheng et al., 2014).

Immunostaining with specific antibodies that recognize presynaptic proteins of excitatory and inhibitory neurons (i.e., vesicular glutamate transporters, vesicular GABA [proteins](#) and transporters) and the postsynaptic density protein-95 kDa (PSD-95) can be applied to enumerate [synaptose](#) [synapse](#) number (Gatto and Broadie, 2010, Akashi et al., 2009). There are commercially available 'synaptogenesis assay kits' that rely on the immunostaining of cells with common synaptic marker proteins such as MAP-2, PSD-95 and synaptophysin. Some other presynaptic (Bassoon) and postsynaptic

(ProSAP1/Shank2) markers have been shown 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).

Some of the same techniques used in rodent studies have been applied to postmortem tissue in humans. In addition, non-invasive, structural neuroimaging techniques in living subjects are also widely used in human studies to assess hippocampal volume using voxel-based morphometry (VBM). With this approach, volume of brain regions is measured by drawing 'regions of interest' on images from brain scans obtained from magnetic resonance imaging (MRI) or positron emission tomography (PET) scans calculating the volume enclosed (Mechelli et al., 2005). These imaging techniques can be applied in rodent models (Powell et al., 2009; Hasegawa et al., 2010; Pirko et al., 2005; Pirko and Johnson, 2008).

It is recognized that most of these biochemical, molecular, cellular, histological and anatomical methods (e.g., morphometrics, protein quantification using different types of cellular staining, immunohistochemistry, and imaging procedures) can also be applied to complex *in vitro* test systems (Pamies et al., 2016; Hartman et al., 2023; Pomeshchik et al., 2020). Human [brain spheres](#) or brain organoids could be developed in three-dimensional cell culture, resembling hippocampus at different stages development (Sakaguchi et al., 2015). These new methods, if assessed as robust, reliable and reproducible, would allow measurement of the KE in a human-relevant test system.

References

[Akashi K, Kakizaki T, Kamiya H, Fukaya M, Yamasaki M, Abe M, Natsume R, Watanabe M, and Sakimura K, 2009. NMDA Receptor GluN2B \(GluR 2/NR2B\) Subunit Is Crucial for Channel Function, Postsynaptic Macromolecular Organization, and Actin Cytoskeleton at Hippocampal CA3 Synapses. The Journal of Neuroscience, 29\(35\):10869 –10882.](#)

[Altman J and Bayer SA, 1990b. Prolonged sojourn of developing pyramidal cells in the intermediate zone of the hippocampus and their settling in the stratum pyramidale. The Journal of comparative neurology, 301\(3\), 343–364. <https://doi.org/10.1002/cne.903010303>](#)

[Altman J and Bayer SA, 1990a. Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. The](#)

Journal of comparative neurology, 301(3), 365–381.
<https://doi.org/10.1002/cne.903010304>

Amaral D and Lavenex P, 2006. "Chapter 3. Hippocampal Neuroanatomy". In: Andersen P, Morris R, Amaral D, Bliss T, O'Keefe J. The Hippocampus Book. Oxford University Press. ISBN 978-0-19-510027-3.

Bongmbaa OYN, Martineza L.A, Elhardta ME, Butlera K, and Tejada-Simona MV, 2011, Modulation of dendritic spines and synaptic function by Rac1: a possible link to Fragile X syndrome pathology. Brain research, 1399, 79–95.
<https://doi.org/10.1016/j.brainres.2011.05.020>

Cheng C, Trzcinski O and Doering LC, 2014. Fluorescent labeling of dendritic spines in cell cultures with the carbocyanine dye "DiI". Frontiers in neuroanatomy, 8, 30.
<https://doi.org/10.3389/fnana.2014.00030>

Dailey ME, and Smith SJ, 1996. The Dynamics of Dendritic Structure in Developing Hippocampal Slices. Journal of Neuroscience, 16 (9) 2983-2994.
<https://doi.org/10.1523/JNEUROSCI.16-09-02983.1996>

Ehrlich I, Klein M, Rumpel S, and Malinow R, 2007. PSD-95 is required for activity-driven synapse stabilization. PNAS, 104; 4181. 104 (10) 4176-4181.
<https://doi.org/10.1073/pnas.0609307104>

Fiala JC, Feinberg M, Popov V, Harris KM. 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. The Journal of neuroscience : the official journal of the Society for Neuroscience, 18(21), 8900–8911.
<https://doi.org/10.1523/JNEUROSCI.18-21-08900.1998>

Garman RH, Fix AS, Jortner BS, Jensen KF, Hardisty JF, Claudio L, Ferenc S. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. II: neuropathology. Environ Health Perspect. 2001 Mar;109 Suppl 1:93-100.

Gatto CL, Broadie K. (2010) Genetic controls balancing excitatory and inhibitory synaptogenesis in neurodevelopmental disorder models. Front Syn Neurosci. 2: 4.

Grabrucker A, Vaida B, Bockmann J, Boeckers TM. (2009) Synaptogenesis of hippocampal neurons in primary cell culture. Cell Tissue Res. 338: 333-341.

Hardy, LR & Redmond, L 2008, 'Translating neuronal activity into dendrite elaboration: Signaling to the nucleus', NeuroSignals, vol. 16. Doi: 10.1159/000111563

Hardy, LR & Redmond, L 2008, 'Translating neuronal activity into dendrite elaboration: Signaling to the nucleus', NeuroSignals, vol. 16.

Harrill JA, Mundy WR, 2011, Quantitative assessment of neurite outgrowth in PC12 cells. Methods Mol Biol., 758:331-48. doi: 10.1007/978-1-61779-170-3_23.

Hartmann J, Henschel N, Bartmann K, Dönmez A, Brockerhoff G, Koch K, Fritsche E. Molecular and Functional Characterization of Different BrainSphere Models for Use in Neurotoxicity Testing on Microelectrode Arrays. Cells. 2023 Apr 27;12(9):1270. doi: 10.3390/cells12091270.

Hasegawa M, Kida I, Wada H. A volumetric analysis of the brain and hippocampus of rats rendered perinatal hypothyroid. Neurosci Lett. 2010 Aug 2;479(3):240-4.

Hevner RF. Layer-specific markers as probes for neuron type identity in human neocortex and malformations of cortical development. J Neuropathol Exp Neurol. 2007 66(2):101-9.

Hinkemeyer M, Itkis OS, Ngo M, Hickmott PW, Ethell IM 2003. Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus. J Cell Biol 2003, 163:1313–1326. Front Mol Neurosci.

Joo, Y., Xue, Y., Wang, Y. et al. Topoisomerase 3 β knockout mice show transcriptional and behavioural impairments associated with neurogenesis and synaptic plasticity. Nat Commun 11, 3143 (2020). <https://doi.org/10.1038/s41467-020-16884-4>

Kier, EL, Kim, JH, Fulbright, K, Bronen, RA. Embryology of the human fetal hippocampus: MR imaging, anatomy, and histology. AJNR Am J Neuroradiol: 1997, 18(3):525-32.

Laeremans, A, Van de Plas, B, Clerens, S, Van den Bergh, G, Arckens, L, Hu, TT. Protein Expression Dynamics During Postnatal Mouse Brain Development. J Exp Neurosci. 2013; 7: 61–74).

Levine ND, Rademacher DJ, Collier TJ, O'Malley JA, Kells AP, San Sebastian W, Bankiewicz KS, Steece-Collier K. (2013) Advances in thin tissue Golgi-Cox impregnation: fast, reliable methods for multi-assay analyses in rodent and non-human primate brain. J Neurosci Methods 213: 214-227.

Mechelli A, Price C, Friston K, Ashburner J (2005) Voxel-Based Morphometry of the Human Brain: Methods and Applications. Curr Med Imaging Rev 1:105-113.

Megias M, Emri Z, Freund TF, Gulyas AI. (2001) Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. Neuroscience 102: 527-540.

Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z., 2002, Abnormal Spine Morphology and Enhanced LTP in LIMK-1 Knockout Mice. Neuron, 35;121-133.

Mody M, Cao Y, Cui Z, Tay KY, Shyong A, Shimizu E, Pham K, Schultz P, Welsh D, Tsien JZ. Genome-wide gene expression profiles of the developing mouse hippocampus. Proc Natl Acad Sci U S A. 2001 Jul 17;98(15):8862-7.

Nieland TJF, Logan DJ, Saulnier J, Lam D, Johnson C, et al. (2014) High Content Image Analysis Identifies Novel Regulators of Synaptogenesis in a High-Throughput RNAi Screen of Primary Neurons. PLoS ONE. 9: e91744.

Odawara A, Katoh H, Matsuda N, Suzuki I. Induction of long-term potentiation and depression phenomena in human induced pluripotent stem cell-derived cortical neurons. Biochem Biophys Res Commun. 2016 Jan 22;469(4):856-62. doi: 10.1016/j.bbrc.2015.12.087.

OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. 2023. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)

OECD. 2007. OECD guidelines for the testing of chemicals/ section 4: Health effects. Test no. 426: Developmental neurotoxicity study. <http://www.oecd.org/dataoecd/20/52/37622194>.

Pamies D, Barreras P, Block K, Makri G, Kumar A, Wiersma D, Smirnova L, Zang C, Bressler J, Christian KM, Harris G, Ming GL, Berlinicke CJ, Kyro K, Song H, Pardo CA, Hartung T, Hogberg HT. A human brain microphysiological system derived from induced pluripotent stem cells to study neurological diseases and toxicity. ALTEX. 2017;34(3):362-376. doi: 10.14573/altex.1609122. Epub 2016 Nov 24. PMID: 27883356; PMCID: PMC6047513.

Pchitskaya E, and Bezprozvanny I, 2020, Dendritic Spines Shape Analysis—Classification or Clusterization? Perspective. Front. Synaptic Neurosci., Front Synaptic Neurosci., 30;12:31.

Pfeiffer T, Poll S, Bancelin S, Angibaud J, Inavalli K, Keppler K, Mittag M, Fuhrmann M, Nägerl V., (2018) Chronic 2P-STED imaging reveals high turnover of dendritic spines in the hippocampus in vivo. eLife, 7, e34700. <https://doi.org/10.7554/eLife.34700>

Pirko I and Johnson AJ,2008. Neuroimaging of demyelination and remyelination models. Current topics in microbiology and immunology, 318, 241–266. https://doi.org/10.1007/978-3-540-73677-6_10

Pirko I, Fricke ST, Johnson AJ, Rodriguez M and Macura SI,2005. Magnetic resonance imaging, microscopy, and spectroscopy of the central nervous system in experimental animals. NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics, 2(2), 250–264. <https://doi.org/10.1602/neurorx.2.2.250>

Pistollato F, de Gyves EM, Carpi D, Bopp SK, Nunes C, Worth A, Bal-Price A. Assessment of developmental neurotoxicity induced by chemical mixtures using an adverse outcome pathway concept. *Environmental health : a global access science source*, 19(1), 23. <https://doi.org/10.1186/s12940-020-00578-x>

Pomeshchik Y, Klementieva O, Gil J, Martinsson I, Hansen MG, de Vries T, Sancho-Balsells A, Russ K, Savchenko E, Collin A, Vaz AR, Bagnoli S, Nacmias B, Rampon C, Sorbi S, Brites D, Marko-Varga G, Kokaia Z, Rezeli M, Gouras GK, Roybon L. Human iPSC-Derived Hippocampal Spheroids: An Innovative Tool for Stratifying Alzheimer Disease Patient-Specific Cellular Phenotypes and Developing Therapies. *Stem Cell Reports*. 2020 Jul 14;15(1):256-273. doi: 10.1016/j.stemcr.2020.06.001. Epub 2020 Jun 25. Erratum in: *Stem Cell Reports*. 2021 Nov 9;16(11):2838. Erratum in: *Stem Cell Reports*. 2023 May 9;18(5):1244-1245.

Powell MH, Nguyen HV, Gilbert M, Parekh M, Colon-Perez LM, Mareci TH and Montie E, 2012. Magnetic resonance imaging and volumetric analysis: novel tools to study the effects of thyroid hormone disruption on white matter development. *Neurotoxicology*, 33(5), 1322–1329. <https://doi.org/10.1016/j.neuro.2012.08.008>

Pré D, Wooten AT, Biesmans S, Hinckley S, Zhou H, Sherman SP, Kakad P, Gearhart J, Bang AG. Development of a platform to investigate long-term potentiation in human iPSC-derived neuronal networks. *Stem Cell Reports*. 2022 Sep 13;17(9):2141-2155. doi: 10.1016/j.stemcr.2022.07.012.

Rizo J and Rosenmund CH, 2008. Synaptic vesicle fusion. *Nature structural & molecular biology*, 15(7), 665–674. <https://doi.org/10.1038/nsmb.1450> .

Runge K, Cardoso C and de Chevigny A, 2020. Dendritic Spine Plasticity: Function and Mechanisms. *Frontiers in synaptic neuroscience*, 12, 36. <https://doi.org/10.3389/fnsyn.2020.00036>

Sakaguchi, H., Kadoshima, T., Soen, M. et al. Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. *Nat Commun* 6, 8896 (2015). <https://doi.org/10.1038/ncomms9896>

Serrano M. E., Kim E, Petrinovic M.M, Turkheimer F and Cash D. 2022. Imaging Synaptic Density: The Next Holy Grail of Neuroscience? *Frontiers in Neuroscience*, Volume 16 - 2022 <https://doi.org/10.3389/fnins.2022.796129>

Shen K. and Cowan CW, 2010. Guidance molecules in synapse formation and plasticity. *Cold Spring Harbor perspectives in biology*, 2(4), a001842. <https://doi.org/10.1101/cshperspect.a001842>

Sudhof TC, 2018. Towards an Understanding of Synapse Formation. *Neuron*, 100(2), 276–293. <https://doi.org/10.1016/j.neuron.2018.09.040>

U.S.EPA. 1998. Health effects guidelines OPPTS 870.6300 developmental neurotoxicity study. EPA Document 712-C-98-239. Office of Prevention Pesticides and Toxic Substances.

Utsunomiya H, Takano k, Okazaki M and Mitsudome A,1999. Development of the temporal lobe in infants and children: analysis by MR-based volumetry. *AJNR. American journal of neuroradiology*, 20(4), 717–723.

West MJ ,1990. "Chapter 2 : Stereological studies of the hippocampus: a comparison of the hippocampal subdivisions of diverse species including hedgehogs, laboratory rodents, wild mice and men". In:Progress in Brain Research. Progress in Brain Research ,83: 13–36.

Zgraggen E, Boitard M, Roman I, Kanemitsu M, Potter G, Salmon P, Vutskits L, Dayer AG, Kiss JZ,2012. Early Postnatal Migration and Development of Layer II Pyramidal Neurons in the Rodent Cingulate/Retrosplenial Cortex, *Cerebral Cortex*, 22(1), 144–157. <https://doi.org/10.1093/cercor/bhr097>

Zhong S, Ding W, Sun L, Lu Y, Dong H, Fan X, Liu Z, Chen R, Zhang S, Ma Q, Tang F, Wu Q and Wang X,2020. Decoding the development of the human hippocampus. *Nature* ,577, 531–536.<https://doi.org/10.1038/s41586-019-1917-5>

Sakaguchi, H., Kadoshima, T., Soen, M. et al. Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. *Nat Commun* 6, 8896 (2015). <https://doi.org/10.1038/ncomms9896>

Pamies D, Barreras P, Block K, Makri G, Kumar A, Wiersma D, Smirnova L, Zang C, Bressler J, Christian KM, Harris G, Ming GL, Berlinicke CJ, Kyro K, Song H, Pardo CA, Hartung T, Hogberg HT. A human brain microphysiological system derived from induced pluripotent stem cells to study neurological diseases and toxicity. *ALTEX*. 2017;34(3):362–376. doi: 10.14573/altex.1609122. Epub 2016 Nov 24. PMID: 27883356; PMCID: PMC6047513.

Pomeshchik Y, Klementieva O, Gil J, Martinsson I, Hansen MG, de Vries T, Sancho-Balsells A, Russ K, Savchenko E, Collin A, Vaz AR, Bagnoli S, Naemias B, Rampon C, Sorbi S, Brites D, Marko-Varga G, Kokaia Z, Rezeli M, Gouras GK, Reybon L. Human iPSC-Derived Hippocampal Spheroids: An Innovative Tool for Stratifying Alzheimer Disease Patient-Specific Cellular Phenotypes and Developing Therapies. *Stem Cell Reports*. 2020 Jul 14;15(1):256–273. doi: 10.1016/j.stemcr.2020.06.001. Epub 2020 Jun 25. Erratum in: *Stem Cell Reports*. 2021 Nov 9;16(11):2838. Erratum in: *Stem Cell Reports*. 2023 May 9;18(5):1244–1245.

Hartmann J, Henschel N, Bartmann K, Dönmez A, Brockerhoff G, Koch K, Fritzsche E. Molecular and Functional Characterization of Different BrainSphere Models for Use in Neurotoxicity Testing on Microelectrode Arrays. *Cells*. 2023 Apr 27;12(9):1270. doi: 10.3390/cells12091270.

Pré D, Wooten AT, Biesmans S, Hinckley S, Zhou H, Sherman SP, Kakad P, Gearhart J, Bang AG. Development of a platform to investigate long term potentiation in human iPSC-derived neuronal networks. *Stem Cell Reports*. 2022 Sep 13;17(9):2141-2155. doi: 10.1016/j.stemcr.2022.07.012.

Odawara A, Katoh H, Matsuda N, Suzuki I. Induction of long-term potentiation and depression phenomena in human induced pluripotent stem cell-derived cortical neurons. *Biochem Biophys Res Commun*. 2016 Jan 22;469(4):856-62. doi: 10.1016/j.bbrc.2015.12.087.

Akashi K, Kakizaki T, Kamiya H, Fukaya M, Yamasaki M, Abe M, Natsume R, Watanabe M, and Sakimura K. 2009. NMDA Receptor GluN2B (GluR 2/NR2B) Subunit Is Crucial for Channel Function, Postsynaptic Macromolecular Organization, and Actin Cytoskeleton at Hippocampal CA3 Synapses. *The Journal of Neuroscience*, 29(35):10869-10882.

Altman J and Bayer SA, 1990a. Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. *The Journal of comparative neurology*, 301(3), 365-381. <https://doi.org/10.1002/cne.903010304>

Altman J and Bayer SA, 1990b. Prolonged sojourn of developing pyramidal cells in the intermediate zone of the hippocampus and their settling in the stratum pyramidale. *The Journal of comparative neurology*, 301(3), 343-364. <https://doi.org/10.1002/cne.903010303>

Amaral D and Lavenex P, 2006. "Chapter 3. Hippocampal Neuroanatomy". In: Andersen P, Morris R, Amaral D, Bliss T, O'Keefe J. *The Hippocampus Book*. Oxford University Press. ISBN 978-0-19-510027-3.

Bongmbaa OYN, Martineza L.A, Elhardta ME, Butlera K, and Tejada Simona MV, 2011, Modulation of dendritic spines and synaptic function by Rac1: a possible link to Fragile X syndrome pathology. *Brain research*, 1399, 79-95. <https://doi.org/10.1016/j.brainres.2011.05.020>

Cheng C, Trzcinski O and Doering LC, 2014. Fluorescent labeling of dendritic spines in cell cultures with the carbocyanine dye "DiI". *Frontiers in neuroanatomy*, 8, 30. <https://doi.org/10.3389/fnana.2014.00030>

Dailey ME, and Smith SJ, 1996. The Dynamics of Dendritic Structure in Developing Hippocampal Slices. *Journal of Neuroscience*, 16 (9) 2983-2994. <https://doi.org/10.1523/JNEUROSCI.16-09-02983.1996>

Ehrlich I, Klein M, Rumpel S, and Malinow R, 2007. PSD-95 is required for activity driven synapse stabilization. *PNAS*, 104; 4181-4186. 104 (10) 4176-4181. <https://doi.org/10.1073/pnas.0609307104>

Fiala JC, Feinberg M, Popov V, Harris KM. 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 18(21), 8900-8911. <https://doi.org/10.1523/JNEUROSCI.18-21-08900.1998>

Garman RH, Fix AS, Jortner BS, Jensen KF, Hardisty JF, Claudio L, Ferenc S. Methods to identify and characterize developmental neurotoxicity for human health risk assessment. II: neuropathology. *Environ Health Perspect*. 2001 Mar;109 Suppl 1:93-100.

Gatto CL, Broadie K. (2010) Genetic controls balancing excitatory and inhibitory synaptogenesis in neurodevelopmental disorder models. *Front Syn Neurosci*. 2: 4.

Grabrucker A, Vaida B, Bockmann J, Boeckers TM. (2009) Synaptogenesis of hippocampal neurons in primary cell culture. *Cell Tissue Res*. 338: 333-341.

Harrill JA, Mundy WR, 2011, Quantitative assessment of neurite outgrowth in PC12 cells. *Methods Mol Biol.*, 758:331-48. doi: 10.1007/978-1-61779-170-3_23.

Hasegawa M, Kida I, Wada H. A volumetric analysis of the brain and hippocampus of rats rendered perinatal hypothyroid. *Neurosci Lett*. 2010 Aug 2;479(3):240-4.

Hevner RF. Layer specific markers as probes for neuron type identity in human neocortex and malformations of cortical development. *J Neuropathol Exp Neurol*. 2007;66(2):101-9.

Hinkemeyer M, Itkis OS, Ngo M, Hickmott PW, Ethell IM. 2003. Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus. *J Cell Biol* 2003, 163:1313-1326. *Front Mol Neurosci*.

Joo, Y., Xue, Y., Wang, Y. et al. Topoisomerase 3 β knockout mice show transcriptional and behavioural impairments associated with neurogenesis and synaptic plasticity. *Nat Commun* 11, 3143 (2020). <https://doi.org/10.1038/s41467-020-16884-4>

Kier, EL, Kim, JH, Fulbright, K, Brønen, RA. Embryology of the human fetal hippocampus: MR imaging, anatomy, and histology. *AJNR Am J Neuroradiol*. 1997, 18(3):525-32.

Laeremans, A, Van de Plas, B, Clerens, S, Van den Berghe, G, Arckens, L, Hu, TT. Protein Expression Dynamics During Postnatal Mouse Brain Development. *J Exp Neurosci*. 2013; 7: 61-74).

Levine ND, Rademacher DJ, Collier TJ, O'Malley JA, Kells AP, San Sebastian W, Bankiewicz KS, Steele Collier K. (2013) Advances in thin tissue Golgi Cox impregnation: fast, reliable methods for multi assay analyses in rodent and non-human primate brain. *J Neurosci Methods* 213: 214-227.

~~Mechelli A, Price C, Friston K, Ashburner J (2005) Voxel Based Morphometry of the Human Brain: Methods and Applications. Curr Med Imaging Rev 1:105-113.~~

~~Megias M, Emri Z, Freund TF, Gulyas AI. (2001) Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. Neuroscience 102: 527-540.~~

~~Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z. 2002. Abnormal Spine Morphology and Enhanced LTP in LIMK-1 Knockout Mice. Neuron, 35:121-133.~~

~~Mody M, Cao Y, Cui Z, Tay KY, Shyong A, Shimizu E, Pham K, Schultz P, Welsh D, Tsien JZ. Genome-wide gene expression profiles of the developing mouse hippocampus. Proc Natl Acad Sci U S A. 2001 Jul 17;98(15):8862-7.~~

~~Nieland TJF, Logan DJ, Saulnier J, Lam D, Johnson C, et al. (2014) High Content Image Analysis Identifies Novel Regulators of Synaptogenesis in a High-Throughput RNAi Screen of Primary Neurons. PLoS ONE. 9: e91744.~~

~~OECD. 2007. OECD guidelines for the testing of chemicals/ section 4: Health effects. Test no. 426: Developmental neurotoxicity study. <http://www.oecd.org/dataoecd/20/52/37622194>.~~

~~OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In Vitro Testing Battery; Series on Testing and Assessment No. 377. 2023. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)~~

~~Pchitskaya E, and Bezprozvanny I, 2020, Dendritic Spines Shape Analysis—Classification or Clusterization? Perspective. Front. Synaptic Neurosci., Front Synaptic Neurosci., 30:12:31.~~

~~Pfeiffer T, Poll S, Bancelin S, Angibaud J, Inavalli K, Keppler K, Mittag M, Fuhrmann M, Nägerl V., (2018) Chronic 2P-STED imaging reveals high turnover of dendritic spines in the hippocampus *in vivo*. *eLife*, 7, e34700. <https://doi.org/10.7554/eLife.34700>~~

~~Pirko I, Fricke ST, Johnson AJ, Rodriguez M and Macura SI, 2005. Magnetic resonance imaging, microscopy, and spectroscopy of the central nervous system in experimental animals. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics*, 2(2), 250-264. <https://doi.org/10.1602/neurorx.2.2.250>~~

~~Pirko I and Johnson AJ, 2008. Neuroimaging of demyelination and remyelination models. *Current topics in microbiology and immunology*, 318, 241-266. https://doi.org/10.1007/978-3-540-73677-6_10~~

Pistollato F, de Gyves EM, Carpi D, Bopp SK, Nunes C, Worth A, Bal Price A. Assessment of developmental neurotoxicity induced by chemical mixtures using an adverse outcome pathway concept. *Environmental health : a global access science source*, 19(1), 23. <https://doi.org/10.1186/s12940-020-00578-x>

Powell MH, Nguyen HV, Gilbert M, Parekh M, Colon Perez LM, Mareci TH and Montie E, 2012. Magnetic resonance imaging and volumetric analysis: novel tools to study the effects of thyroid hormone disruption on white matter development. *Neurotoxicology*, 33(5), 1322–1329. <https://doi.org/10.1016/j.neuro.2012.08.008>

Hardy, LR & Redmond, L 2008, 'Translating neuronal activity into dendrite elaboration: Signaling to the nucleus', *NeuroSignals*, vol. 16.

Rizo J and Rosenmund CH, 2008. Synaptic vesicle fusion. *Nature structural & molecular biology*, 15(7), 665–674. <https://doi.org/10.1038/nsmb.1450>.

Runge K, Cardoso C and de Chevigny A, 2020. Dendritic Spine Plasticity: Function and Mechanisms. *Frontiers in synaptic neuroscience*, 12, 36. <https://doi.org/10.3389/fnsyn.2020.00036>

Serrano M. E., Kim E, Petrinovic M.M, Turkheimer F and Cash D. 2022. Imaging Synaptic Density: The Next Holy Grail of Neuroscience? *Frontiers in Neuroscience*, Volume 16 2022 <https://doi.org/10.3389/fnins.2022.796129>

Shen K. and Cowan CW, 2010. Guidance molecules in synapse formation and plasticity. *Cold Spring Harbor perspectives in biology*, 2(4), a001842. <https://doi.org/10.1101/cshperspect.a001842>

Sudhof TC, 2018. Towards an Understanding of Synapse Formation. *Neuron*, 100(2), 276–293. <https://doi.org/10.1016/j.neuron.2018.09.040>

U.S.EPA. 1998. Health effects guidelines OPPTS 870.6300 developmental neurotoxicity study. EPA Document 712-C-98-239. Office of Prevention Pesticides and Toxic Substances.

Utsunomiya H, Takano k, Okazaki M and Mitsudome A, 1999. Development of the temporal lobe in infants and children: analysis by MR-based volumetry. *AJNR. American journal of neuroradiology*, 20(4), 717–723.

West MJ, 1990. "Chapter 2 : Stereological studies of the hippocampus: a comparison of the hippocampal subdivisions of diverse species including hedgehogs, laboratory rodents, wild mice and men". In: *Progress in Brain Research*. *Progress in Brain Research*, 83: 13–36.

~~Zgraggen E, Boitard M, Roman I, Kanemitsu M, Potter G, Salmon P, Vutskits L, Dayer AG, Kiss JZ, 2012. Early Postnatal Migration and Development of Layer II Pyramidal Neurons in the Rodent Cingulate/Retrosplenial Cortex, *Cerebral Cortex*, 22(1), 144-157. <https://doi.org/10.1093/cercor/bhr097>~~

~~Zhong S, Ding W, Sun L, Lu Y, Dong H, Fan X, Liu Z, Chen R, Zhang S, Ma Q, Tang F, Wu Q and Wang X, 2020. Decoding the development of the human hippocampus. *Nature*, 577, 531-536. <https://doi.org/10.1038/s41586-019-1917-5>~~

Abbreviations

AOPs	Adverse outcome pathways
AOP Wiki	Collaborative Adverse Outcome Pathway Wiki
DNT	Developmental Neurotoxicity
DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
EEG	Electroencephalography
EFSA	European Food Safety Agency
EPA	Environmental Protection Agency
EPSP	Excitatory Synaptic Transmission
fMRI	Functional magnetic resonance imaging
IATA	Integrated Approach to Testing and Assessment
IPSCs	Inhibitory synaptic currents
KE	Key Event
KERs	Key Event Relationship
LTP	Long-term potentiation
MIE	Molecular initiating event
NIS	Sodium Iodide Symporter
OECD	Organisation for Economic Co-operation and Development

PSD-95	Postsynaptic density protein-95 kDa
PET	Positron emission tomography
SV	Synaptic vesicle
TEM	Transmission electron microscope
TH	Thyroid Hormone
TR	Thyroid Receptor
TPO	Thyroperoxidase
VBM	voxel-based morphometry
WG	Working Group

KE5: KE758: Hippocampal physiology, altered

KE 758 has been extracted from the AOP wiki on October 10th. Changes proposed by EFSA WG were discussed and agreed with the KE 757 developers in various WG meetings.

Event: 758

Key Event Title

Hippocampal Physiology, Altered

Short name: Hippocampal Physiology, Altered

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

brain

Key Event Components

Process	Object	Action
<u>C</u> hemical synaptic transmission	synapse	abnormal

Key Event Overview

AOPs Including This Key Event

AOP Name	Role of event in AOP	Point of Contact	Author Status	OECD Status
Thyroperoxidase(TPO) Inhibition and Altered Neurodevelopment	KeyEvent	Kevin Crofton (send email)	Open for citation & comment	WPHA/WNT Endorsed
Nuclear receptor induced Thyroid Hormone(TH) Catabolism and Developmental Hearing Loss	KeyEvent	Katie Paul Friedman (send email)	Open for adoption	Under Development
Sodium Iodide Symporter (NIS) and Cognitive Dysfunction	KeyEvent	Mary Gilbert (send email)	Under Development: Contributions and Comments Welcome	
Transthyretin interference	KeyEvent	Kristie Sullivan (send email)	Under Development: Contributions and Comments Welcome	Under Development
Thyroid Receptor (TR) Antagonism and DNT	KeyEvent	Kevin Crofton (send email)	Under development: Not open for	Under Development

AOP Name	Role of event in AOP	Point of Contact	Author Status	OECD Status
			comment. Do not cite	

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
human	<i>Homo sapiens</i>	Moderate	NCBI
rat	<i>Rattus norvegicus</i>	High	NCBI
mouse	<i>Mus musculus</i>	High	NCBI

Life Stages

Life stage	Evidence
During brain development	High

Sex Applicability

Term	Evidence
Female	High
Male	High

KE Developmental Strategy

KE5 was originally created as part of an evidence-based AOP-informed IATA developmental neurotoxicity hazard characterization of deltamethrin. This IATA case study was developed to support human health risk assessment of deltamethrin and as proof of concept on the applicability of the data provided in the Developmental

Neurotoxicity In vitro Battery. It was applied to provide a mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD., 2023). Using systematic searches and expert knowledge the initial MiE and KE descriptions and evidence were updated by an EFSA Working Group. This was followed by a search of existing content in the AOP Wiki to determine whether analogous AOPs and/or synonymous KEs or KERs may already exist in the knowledgebase. After an expert assessment KE5 developed by the EFSA WG was combined with KE758 and [finalthe final](#) text agreed with both KEs developers.

Key Event Description

The hippocampus functions as a highly integrated and organized communication and information processing network with millions of interconnections among its constitutive neurons. Neurons in the hippocampus and throughout the brain transmit and receive information largely through chemical transmission across the synaptic cleft, the space where the specialized ending of the presynaptic axon terminus of the transmitting neuron meets the specialized postsynaptic region of the neuron that is receiving that information (Kandell et al., 2012).

During development (see KE 657: Hippocampal anatomy, Altered), as neurons reach their final destination and extend axonal processes, early patterns of electrical synaptic activity emerge in the hippocampus. These are large fields of axonal innervation of broad synaptic target sites that are replaced by more elaborate, but highly targeted and refined axonal projections and synaptic connectivity brought about by activity-dependent synaptic stabilization, pruning, or synapse elimination. This is a classic case of the interaction between physiological and anatomical development, where anatomy develops first, and is 'reshaped' by physiological function (Kutsarova et al., 2017).

In the rat, excitatory processes are fully mature in area CA1 of hippocampus within 2 weeks of birth with inhibitory processes lagging begin by several weeks (Muller et al., 1989; Michelson and Lothman, 1988; Harris and Teyler, 1984). In hippocampal slices, inhibitory function in area CA1 field is first seen on postnatal day 5, increasing in strength at postnatal day 12 through 15. *In vivo* studies fail to detect inhibition until postnatal day 18 with steady increase thereafter to adult levels by postnatal day 28. Synaptic plasticity in the form of long-term potentiation (LTP) is absent in the very young animal, only emerging about postnatal day 14, appearing to require the stability of both excitatory and inhibitory function to be established (Muller et al., 1989; Bekenstein and Lothman, 1991). These features of the maturation of hippocampal physiology are paralleled in dentate gyrus, but as with anatomical indices in the rat, the development of these physiological parameters lag behind the CA1 by about 1 week. As described in structural development in KE4, a very similar pattern of maturation occurs in the human developing hippocampus, but with a trajectory that is largely complete before birth.

How It Is Measured or Detected

In animals, synaptic function in the hippocampus has been examined with imaging techniques, but more routinely, electrical field potentials recorded in two subregions of the hippocampus, area CA1 and dentate gyrus, have been assessed *in vivo* or *in vitro*. Field potentials recorded in both regions of the hippocampus reflect the summed synaptic response of a population of neurons following direct stimulation of input pathways across a monosynaptic connection. Changes in response amplitude due to chemical perturbations and other stressors (e.g., chemical exposures, nutritional deficits, gene knockouts) is evidence of altered synaptic function. This can be measured *in vitro*, *in vivo*, or in hippocampal slices taken from treated animals (Gilbert and Burdette, 1995). The most common physiological measurements used to assess the function of the hippocampus are excitatory synaptic transmission, inhibitory synaptic transmission, and synaptic plasticity in the form of LTP.

Excitatory Synaptic Transmission: Two measures, the excitatory postsynaptic potential (EPSP) and the population spike are derived from the compound field potential at increasing stimulus strengths. The function described by the relationship of current strength (input, I) and evoked response (output, O), the I-O curve is the measure of excitatory synaptic transmission (Gilbert and Burdette, 1995).

Inhibitory Synaptic Transmission: Pairs of stimulus pulses delivered in close temporal proximity are used to probe the integrity of inhibitory synaptic transmission. The response evoked by the second pulse of the pair at brief intervals (<30 msec) arrives during the activation of feedback inhibitory loops in the hippocampus. An alteration in the degree of suppression to the 2nd pulse of the pair reflects altered inhibitory synaptic function (Gilbert and Burdette, 1995).

Long Term Potentiation (LTP): LTP is widely accepted to be a major component of the cellular processes that underlie learning and memory (Malenka and Bear, 2004; Bramham and Messaoudi, 2005). LTP represents, at the synapse and molecular level, the coincident firing of large numbers of neurons that are engaged during a learning event. The persistence of LTP emulates the duration of the memory. Synaptic plasticity in the form of LTP is assessed by delivering trains of high frequency stimulation to induce a prolonged augmentation of synaptic response. Probe stimuli at midrange stimulus strengths are delivered before and after application of LTP-inducing trains. The degree of increase in EPSP and PS amplitude to the probe stimulus after train application, and the duration of the induced synaptic enhancement are metrics of LTP. Additionally, contrasting I-O functions of excitatory synaptic transmission before and after (hours to days) LTP is induced is also a common measure of LTP maintenance (Bramham and Messaoudi, 2005; Kandell et al., 2012; Malenka and Bear, 2004). LTP has been assessed also using *in vitro* neuronal networks (Odawara et al., 2016; Pre et al., 2022).

Excitatory and inhibitory synaptic currents (EPSCs and IPSCs) can also be measured in single cells, mostly *ex vivo* within slices of hippocampus using intracellular and patch clamp techniques as described in previous KEs. These same outputs can evaluate the integrity of synaptic transmission and synaptic plasticity.

Synaptic function in the human hippocampus has been assessed using electroencephalography (EEG) and functional neuroimaging techniques (Clapp et al., 2012). EEG is a measure of electrical activity over many brain regions but primarily from the cortex using small flat metal discs (electrodes) placed over the surface of the skull. It is a readily available test that provides evidence of how the brain functions over time. Functional magnetic resonance imaging or functional MRI (fMRI) uses MRI technology to measure brain activity by detecting associated changes in blood flow. This technique relies on the fact that cerebral blood flow and neuronal activation are coupled. Positron emission tomography (PET) is a functional imaging technique that detects pairs of gamma rays emitted indirectly by a radionuclide (tracer) injected into the body (Tietze, 2012; McCarthy, 1995). Like fMRI, PET scans indirectly measure blood flow to different parts of the brain – the higher the blood flow, the greater the activation (McCarthy, 1995). These techniques have been widely applied in clinical and research settings to assess learning and memory in humans and can provide information targeted to hippocampal functionality (McCarthy, 1995; Smith and Jonides, 1997; Willoughby et al., 2014; Wheeler et al., 2015; Gilbert et al., 1998).

Assays of this type are fit for purpose, have been well accepted in the literature, and are reproducible across laboratories. The assay directly measures the key event of altered neurophysiological function.

Domain of Applicability

The majority of Most of the evidence for this key event comes from work in rodent species (i.e., rat, mouse). There is a moderate amount of evidence from other species, including humans (Clapp et al., 2012).

References

Bekenstein JW, Lothman EW. An *in vivo* study of the ontogeny of long-term potentiation (LTP) in the CA1 region and in the dentate gyrus of the rat hippocampal formation. *Brain Res Dev Brain Res.* 1991 Nov 19;63(1-2):245-

Bramham CR, Messaoudi E (2005) BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Prog Neurobiol* 76:99-125.

Clapp WC, Hamm JP, Kirk IJ, Teyler TJ. Translating long-term potentiation from animals to humans: a novel method for noninvasive assessment of cortical plasticity. *Biol Psychiatry.* 2012 Mar 15;71(6):496-502.

Gilbert, M.E. and Burdette, L.J. (1995). Hippocampal Field Potentials: A Model System to Characterize Neurotoxicity. In *Neurotoxicology: Approaches and Methods*. L.W Chang and W. Slikker (Eds). Academic Press:New York, 183-204.

Gilbert ME, Mack CM. Chronic lead exposure accelerates decay of long-term potentiation in rat dentate gyrus in vivo. *Brain Res.* 1998 Apr 6;789(1):139-49.

Harris KM, Teyler TJ. Developmental onset of long-term potentiation in area CA1 of the rat hippocampus. *J Physiol.* 1984. 346:27-48.

Kandell, E., Schwartz, J., Siegelbaum, A. and Hudspeth, A.J. (2012) *Principles of Neural Science*, 5th Edition. Elsevier, North Holland.

Kutsarova E, Munz M, Ruthazer ES. Rules for Shaping Neural Connections in the Developing Brain. *Front Neural Circuits.* 2017 Jan 10;10:111. doi: 10.3389/fncir.2016.00111.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. *Neuron* 44:5-21.

McCarthy, G. (1995) Review: Functional Neuroimaging and Memory. *The Neuroscientist*, 1:155-163.

Michelson HB, Lothman EW. An in vivo electrophysiological study of the ontogeny of excitatory and inhibitory processes in the rat hippocampus. *Brain Res Dev Brain Res.* 1989 May 1;47(1):113-22.

Muller D, Oliver M, Lynch G. Developmental changes in synaptic properties in hippocampus of neonatal rats. *Brain Res Dev Brain Res.* 1989 Sep 1;49(1):105-14.

OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. 2023. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)

Smith, E and Jonides, J. (1997). Working Memory: A View from Neuroimaging. *Cognitive Psychology*, 33:5-42.

Tietze, KJ. (2012). Review of Laboratory and Diagnostic Tests- Positron Emission Tomography. In *Clinical Skills for Pharmacists*, 3rd Edition, pp 86-122.

Wheeler SM, McLelland VC, Sheard E, McAndrews MP, Rovet JF (2015) Hippocampal Functioning and Verbal Associative Memory in Adolescents with Congenital Hypothyroidism. *Front Endocrinol (Lausanne)* 6:163.

Willoughby KA, McAndrews MP, Rovet JF (2014) Effects of maternal hypothyroidism on offspring hippocampus and memory. *Thyroid* 24:576-584.

Abbreviations

AOPs	Adverse outcome pathways
AOP Wiki	Collaborative Adverse Outcome Pathway Wiki
DNT	Developmental Neurotoxicity
DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
EEG	Electroencephalography
EFSA	European Food Safety Agency
EPA	Environmental Protection Agency
EPSP	Excitatory Synaptic Transmission
EPSCs	Excitatory synaptic currents
fMRI	Functional magnetic resonance imaging
IATA	Integrated Approach to Testing and Assessment
IPSCs	Inhibitory synaptic currents
KE	Key Event
KERs	Key event relationships
LTP	Long-term potentiation
MIE	Molecular initiating event
NIS	Sodium Iodide Symporter
OECD	Organisation for Economic Co-operation and Development
PET	Positron emission tomography
SV	Synaptic vesicle
TEM	Transmission electron microscope

TH	Thyroid Hormone
TR	Thyroid Receptor
TPO	Thyroperoxidase
WG	Working Group

AO: KE402: Cognitive Function, Decreased

AO 402 has been extracted from the AOP wiki on October 10th. Changes proposed by EFSA WG were discussed and agreed with the AO 402 developers in various WG meetings.

Short name: Cognitive function, decreased.

Cognitive Function, Decreased

Biological Context

- Level of Biological Organization: Individual
- Cell term: blank
- Organ term: brain

Key events components

Process	Object	Action
learning or memory		decreased
cognition		decreased

Key Event Overview

Other AOPs that use this KE

AOP Name	Role of event in AOP	Point of Contact	Author Status	OECD Status
TPO Inhibition and Altered Neurodevelopment	AO	Kevin Crofton	Open for citation & comment	WPHA/WNT Endorsed
NIS and Neurodevelopment	AO	Kevin Crofton	Not under active development	

NIS and Cognitive Dysfunction	AO	Mary Gilbert	Under Development: Contributions and Comments Welcome	
Transthyretin interference	AO	Kristie Sullivan	Under Development: Contributions and Comments Welcome	Under Development
TR Antagonism and DNT	AO	Kevin Crofton	Under development: Not open for comment. Do not cite	Under Development
Organo-Phosphate Chemicals leading to impaired cognitive function	AO	SAROJ AMAR	Under development: Not open for comment. Do not cite	

Taxonomic Applicability

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

Life Stages

Life stage	Evidence
All life stages	High

Sex Applicability

Term	Evidence
Male	High
Female	High

AO Developmental Strategy

AO was originally created as part of an evidence-based AOP-informed IATA developmental neurotoxicity hazard characterization of deltamethrin. This IATA case study was developed to support human health risk assessment of deltamethrin and as proof of concept on the applicability of the data provided in the Developmental Neurotoxicity In vitro Battery. It was applied to provide a mechanistic understanding of toxicity pathways for regulatory decision making (DNT IVB OECD., 2023). Using systematic searches and expert knowledge the initial MiE and KE descriptions and evidence were updated by an EFSA Working Group. This was followed by a search of existing content in the AOP Wiki to determine whether analogous AOPs and/or synonymous KEs or KERs may already exist in the knowledge base. After an expert assessment KE5 developed by the EFSA WG was combined with AO: KE402 and final text agreed with both KEs developers.

KE Description

Learning and memory depend upon the coordinated action of different brain regions and neurotransmitter systems constituting functionally integrated neural networks (D'Hooge and DeDeyn, 2001). Among the many brain areas engaged in the acquisition or retrieval of a learned event, the hippocampal-based memory systems have received the most study. The main learning areas and pathways are similar in rodents and primates, including man (Eichenbaum, 2000; Stanton and Spear, 1990; Squire, 2004; Gilbert., 2006).

In humans, the hippocampus is involved in recollection of an event's rich spatial-temporal contexts and distinguished from simple semantic memory which is memory of a list of facts (Burgess et al., 2000). Hemispheric specialization has occurred in humans, with the left hippocampus specializing in verbal and narrative memories (i.e., context-dependent episodic or autobiographical memory) and the right hippocampus, more prominently engaged in visuo-spatial memory (i.e., memory for locations within an environment). The hippocampus is particularly critical for the formation of episodic memory, and autobiographical memory tasks have been developed to specifically probe these functions (Eichenbaum, 2000; Willoughby et al., 2014). In rodents, there is obviously no verbal

component in hippocampal memory, but reliance on the hippocampus for spatial, temporal and contextual memory function has been well documented. Spatial memory deficits and fear-based context learning paradigms engage the hippocampus, amygdala, and prefrontal cortex (Eichenbaum, 2000; Shors et al., 2001; Samuels et al., 2011; Vorhees and Williams, 2014; D'Hooge and DeDeyn, 2001; Lynch, 2004; O'Keefe and Nadal, 1978). These tasks are impaired in animals with hippocampal dysfunction (O'Keefe and Nadal, 1978; Morris and Frey, 1987; Gilbert et al., 2016).

How It Is Measured or Detected

In rodents, a variety of tests of learning and memory have been used to probe the integrity of hippocampal function. These include tests of spatial learning like the radial arm maze (RAM), the Barnes maze, and most commonly, the Morris water maze (MWM). Tests ~~of novelty~~ such as novel object recognition, and fear-based context learning are also sensitive to hippocampal disruption. Finally, trace fear conditioning which incorporates a temporal component upon traditional amygdala-based fear learning engages the hippocampus. The text below provides brief descriptions of the ~~most commonly used~~most used tasks.

- 1) RAM, Barnes Maze, and MWM are examples of spatial tasks in which animals are required to learn: the location of a food reward (RAM); an escape hole to enter a preferred dark tunnel from a brightly lit open field area (Barnes maze); or a hidden platform submerged below the surface of the water in a large tank of water (MWM) (Vorhees and Williams, 2014).
- 2) Novel Object Recognition (NOR) and its variants are widely used in neuroscience, although their suitability for safety assessment remains unclear (Vorhees and Williams, 2024). NOR and novel place recognition (NPR) are examples of 'incidental learning' and rely on the dorsal hippocampus. They are simple tasks and are used to probe recognition memory. Two objects are presented to animals in an open field on trial 1, and animals are allowed time to briefly explore them. On trial 2, one object is replaced with a novel object and time spent interacting with the novel object is taken as evidence of memory retention (i.e., one of these objects is familiar, the other is novel (Cohen and Stackman, 2015). In novel place recognition, the objects are shifted to a location within the arena. Compared to tests of spatial learning, the learning event is transient, the results often variable, and the test has a very narrow dynamic range.

- 3) Contextual Fear Conditioning is a hippocampal based learning task in which animals are placed in a novel environment and allowed to explore for several minutes before delivery of an aversive stimulus, typically a mild foot shock. Upon reintroduction to this

same environment in the future (typically 24-48 hours after original training), animals will limit their exploration, the context of this chamber being associated with an aversive event (unconditional stimulus, US). The degree of suppression of activity after training is taken as evidence of retention, i.e., memory (Curzon et al., 2009).

4) Trace Fear Conditioning. Standard fear conditioning paradigms require animals to make an association between a neutral conditioning stimulus (CS, e.g., a light or a tone) and an aversive stimulus (US, e.g., a foot_shock). The unconditioned response (CRUR) that is elicited upon delivery of the foot_shock US is freezing behavior. With repetition of CS/US delivery, the previously neutral stimulus comes to elicit the freezing response. This type of learning is dependent on the amygdala, a brain region associated with, but distinct from the hippocampus. Introducing a brief delay between presentation of the neutral CS and the aversive US, a trace period, requires the engagement of the amygdala and the hippocampus (Shors et al., 2004).

Most methods used in animals are well established in the published literature, and many have been engaged to evaluate the effects of developmental neurotoxicants. The US EPA and OECD Developmental Neurotoxicity (DNT) Guidelines (OCSPP 870.6300 or OECD 426) both require testing of learning and memory (USEPA, 1998; OECD, 2007). These DNT Guidelines have been deemed valid to identify DNT and adverse neurodevelopmental outcomes (Makris et al., 2009).

A variety of standardized learning and memory tests have been developed for human neuropsychological testing. These include episodic autobiographical memory, word pair recognition memory; object location recognition memory. Some components of these tests have been incorporated in general tests of adult intelligence (IQ) such as the Wechsler Adult Intelligence Scale (WAIS) which calculates four composite scores that examine various domains within an individual's overall cognitive ability: Verbal Comprehension Index (VCI), Perceptual Reasoning Index (PRI), Working Memory Index (WMI), and Processing Speed Index (PSI) (Climie and Rostad, 2011). Modifications have been made and norms developed for incorporating tests of learning and memory in children. Examples of some of these tests include:

- 1) Rey Osterieth Complex Figure (RCFT) which probes a variety of functions including visuospatial abilities, memory, attention, planning, and working memory (Shin et al., 2006).
- 2) Children's Auditory Verbal Learning Test (CAVLT) is a free recall of presented word lists that yields measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak 1994; Talley, 1986).
- 3) Continuous Visual Memory Test (CVMT) measures visual learning and memory. It is a free recall of presented pictures/objects rather than words but that yields similar

measures of Immediate Memory Span, Level of Learning, Immediate Recall, Delayed Recall, Recognition Accuracy, and Total Intrusions. (Lezak, 1984; 1994).

- 4) Story Recall from Wechsler Memory Scale (WMS) Logical Memory Test Battery, a standardized neuropsychological test designed to measure memory functions (Lezak, 1994; Talley, 1986).
- 5) Autobiographical memory (AM) is the recollection of specific personal events in a multifaceted higher order cognitive process. It includes episodic memory- remembering of past events specific in time and place, in contrast to semantic autobiographical memory is the recollection of personal facts, traits, and general knowledge. Episodic AM is associated with greater activation of the hippocampus and a later and more gradual developmental trajectory. Absence of episodic memory in early life (infantile amnesia) is thought to reflect immature hippocampal function (Herold et al., 2015; Fivush, 2011).
- 6) Staged AM Task. In this version of the AM test, children participate in a staged event involving a tour of the hospital, perform a series of tasks (counting footprints in the hall, identifying objects in wall display, buying lunch, watched a video). It is designed to contain unique event happenings, place, time, visual/sensory/perceptual details. Four to five months later, interviews are conducted using Children's Autobiographical Interview and scored according to standardized scheme (Willoughby et al., 2014).

Domain of Applicability

Basic forms of learning behavior such as habituation have been found in many taxa from worms to humans (Alexander, 1990). More complex cognitive processes such as executive function likely reside only in higher mammalian species such as non-human primates and humans.

Regulatory Significance of the Adverse Outcome

A prime example of impairments in cognitive function as the adverse outcome for regulatory action is developmental lead exposure and IQ function in children (Bellinger, 2012). In addition, testing for the impact of chemical exposures on cognitive function, often including spatially-mediated behaviors, is an integral part of both EPA and OECD developmental neurotoxicity guidelines (USEPA, 1998; OECD, 2007).

References

Alexander RD (1990) Epigenetic rules and Darwinian algorithms: The adaptive study of learning and development. *Ethology and Sociobiology* 11:241-303.

Bellinger DC (2012) A strategy for comparing the contributions of environmental chemicals and other risk factors to neurodevelopment of children. *Environ Health Perspect* 120:501-507.

Burgess N (2002) The hippocampus, space, and viewpoints in episodic memory. *Q J Exp Psychol A* 55:1057-1080.

Climie, E. A., & Rostad, K. (2011). Test Review: Wechsler Adult Intelligence Scale. *Journal of Psychoeducational Assessment*, 29(6), 581–586. <https://doi.org/10.1177/0734282911408707>

Cohen, SJ and Stackman, RW. (2015). Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behav. Brain Res.* 285: 105-1176.

Curzon P, Rustay NR, Browman KE. Cued and Contextual Fear Conditioning for Rodents. In: Buccafusco JJ, editor. *Methods of Behavior Analysis in Neuroscience*. 2nd edition. Boca Raton (FL): CRC Press/Taylor & Francis; 2009

D'Hooge R, De Deyn PP (2001) Applications of the Morris water maze in the study of learning and memory. *Brain Res Brain Res Rev* 36:60-90.

Eichenbaum H (2000) A cortical-hippocampal system for declarative memory. *Nat Rev Neurosci* 1:41-50.

Fivush R. The development of autobiographical memory. *Annu Rev Psychol.* 2011. 62:559-82.

Gilbert ME, Sanchez-Huerta K, Wood C (2016) Mild Thyroid Hormone Insufficiency During Development Compromises Activity-Dependent Neuroplasticity in the Hippocampus of Adult Male Rats. *Endocrinology* 157:774-787.

Gilbert ME, Sui L (2006) Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. *Brain Res* 1069:10-22.

Herold, C, Lässer, MM, Schmid, LA, Seidl, U, Kong, L, Fellhauer, I, Thomann, PA, Essig, M and Schröder, J. (2015). Neuropsychology, Autobiographical Memory, and Hippocampal Volume in "Younger" and "Older" Patients with Chronic Schizophrenia. *Front. Psychiatry*, 6: 53.

Lezak MD (1984) Neuropsychological assessment in behavioral toxicology--developing techniques and interpretative issues. *Scand J Work Environ Health* 10 Suppl 1:25-29.

Lezak MD (1994) Domains of behavior from a neuropsychological perspective: the whole story. *Nebr Symp Motiv* 41:23-55.

Lynch, M.A. (2004). Long-Term Potentiation and Memory. *Physiological Reviews*. 84:87-136.

Makris SL, Raffaele K, Allen S, Bowers WJ, Hass U, Alleva E, Calamandrei G, Sheets L, Amcoff P, Delrue N, Crofton KM. A retrospective performance assessment of the developmental neurotoxicity study in support of OECD test guideline 426. *Environ Health Perspect*. 2009 Jan;117(1):17-25.

Morris RG, Frey U. Hippocampal synaptic plasticity: role in spatial learning or the automatic recording of attended experience? *Philos Trans R Soc Lond B Biol Sci*. 1997 Oct 29;352(1360):1489-503. Review

O'Keefe, J. and Nadel, L. (1978). *The Hippocampus as a Cognitive Map*. Oxford: Oxford University Press.

OECD. 2007. OECD guidelines for the testing of chemicals/ section 4: Health effects. Test no. 426: Developmental neurotoxicity study. www.Oecd.Org/dataoecd/20/52/37622194.Pdf [accessed May 21, 2012].

OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. 2023. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)

Samuels BA, Hen R (2011) Neurogenesis and affective disorders. *Eur J Neurosci* 33:1152-1159.

Shin, MS, Park, SY, Park, SR, Oeol, SH and Kwon, JS. (2006). Clinical and empirical applications fo the Rey-Osterrieth complex figure test. *Nature Protocols*, 1: 892-899.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. *Nature* 410:372-376.

Squire LR (2004) Memory systems of the brain: a brief history and current perspective. *Neurobiol Learn Mem* 82:171-177.

Stanton ME, Spear LP (1990) Workshop on the qualitative and quantitative comparability of human and animal developmental neurotoxicity, Work Group I report: comparability of

measures of developmental neurotoxicity in humans and laboratory animals. *Neurotoxicol Teratol* 12:261-267.

Talley, JL. (1986). Memory in learning disabled children: Digit span and eh Rey Auditory verbal learning test. *Archives of Clinical Neuropsychology*, Elsevier.

U.S.EPA. 1998. Health effects guidelines OPPTS 870.6300 developmental neurotoxicity study. EPA Document 712-C-98-239. Office of Prevention Pesticides and Toxic Substances.

Vorhees CV, Williams MT (2014) Assessing spatial learning and memory in rodents. *ILAR J* 55:310-332.

Vorhees, C and Williams M. Tests for Learning and Memory in Rodent Regulatory Studies. *Current Research in Toxicology*, 2024, [in press Curr Res Toxicol. 2024; 6: 100151. doi: 10.1016/j.crtox.2024.100151](#)

Willoughby KA, McAndrews MP, Rovet JF. Accuracy of episodic autobiographical memory in children with early thyroid hormone deficiency using a staged event. *Dev Cogn Neurosci*. 2014. 9:1-11.

Glossary [and/or] Abbreviations

AM	Autobiographical memory
AOPs	Adverse outcome pathways
AOP Wiki	Collaborative Adverse Outcome Pathway Wiki
CAVLT	Children's Auditory Verbal Learning Test
CVMT	Continuous Visual Memory Test
CRUR	Unconditioned response
DNT	Developmental Neurotoxicity

DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
EFSA	European Food Safety Agency
EPA	Environmental Protection Agency
IATA	Integrated Approach to Testing and Assessment
KE	Key Event
KERs	Key event relationships
MIE	Molecular initiating event
MWM	Morris water maze
NIS	Sodium Iodide Symporter
NOR	Novel Object Recognition
NPR	Novel place recognition
OECD	Organisation for Economic Co-operation and Development
PRI	Perceptual Reasoning Index
PSI	Processing Speed Index
RAM	Radial arm maze
RCFT	Rey Osterieth Complex Figure
TR	Thyroid Receptor
TPO	Thyroperoxidase
VCI	Verbal Comprehension Index
WAIS	Wechsler Adult Intelligence Scale
WMI	Working Memory Index
WMS	Wechsler Memory Scale

WG

Working Group

Key Events Relationships

KER1: KER2605 Binding to voltage-gated sodium channel leads to aAltered kinetics of sodium channel

Key Event Relationship Title

Upstream event: Binding to voltage-gated sodium channel

Downstream event: Altered kinetics of sodium channel

Key Event Relationship Overview

AOPs Referencing Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence
Vertebrates	Vertebrates	HIGH
Invertebrates	Invertebrates	HIGH

Sex Applicability

Sex	Evidence
Male	HIGH
Female	HIGH

Life Stage Applicability

Term	Evidence
All life stages	HIGH

Key Event Relationship Description

VGSCs are critical in generation and conduction of electrical signals in multiple excitable tissues. Various chemicals and agents can interfere with VGSC function through several mechanisms, leading to alterations of VGSC function. The type of alteration depends on how the compound interacts with the VGSC. Hydrophobic anesthetics may bind within the hydrophobic zone in the pore, blocking the channel in the closed state, while hydrophilic anesthetics may bind to the pore on an intracellular site blocking the channel in the inactivation phase. For the latter, high or low dissociation rates affect anesthetic potency in situations of high or low frequency firing, respectively. Other chemicals, like the antiepileptic carbamazepine or the *α*Amyotrophic lateral sclerosis-treatment drug riluzole, bind to the voltage sensors in the channels and thereby shift the voltage dependency of their open/closed configurations. In contrast, toxins like tetrodotoxin (TTX) bind to the extracellular regions of VGSCs, block the passage of ions and cannot be removed by either changing the membrane voltage or the gating of the channel (Eijkelkamp et al. 2012; Catterall 2007). The pyrethroid insecticides also bind to VGSCs but in a manner that slows both activation and deactivation of the gate and results in a more hyperpolarized membrane potential and in higher firing rates (Eijkelkamp et al., 2012; Trainer et al., 1997; O'Reilly et al., 2006; Meyer et al., 2008; Soderlund et al., 2002).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is strong. It is a well-accepted fact that ion channels are integral membrane proteins that control the passage of various ions (Na^+ , K^+ , Ca^{2+} , Cl^-) across *lipid*-membranes in cells. The direction of ion transport through an open ion channel is governed by the electrochemical gradient for the particular ion species across the membrane in question. There is overwhelming evidence that binding of a chemical to a VGSC alters sodium channels kinetics. This is well supported by studies in which individual channel residues are mutated, and these mutations alter the ability of different

chemicals to interact with the sodium channel to alter its gating kinetics (e.g. Vais et al., 2000; 2001). The stereospecific nature of effects of many different compounds on VGSC function further supports that specific binding leads to alterations in the kinetics of the channel (Soderlund 1985; Brown et al., 1988; Narahashi 1982).

Empirical Evidence

The empirical evidence for this KER is strong. A wide variety of natural-occurring toxins have been demonstrated to interact with VGSCs and alter function of the channel. These toxins include TTX, the poison in fugu (pufferfish); scorpion and sea anemone toxins, brevotoxins from dinoflagellates, ciguatoxins and some conotoxins from fish-hunting snails. VGSCs possess six or more distinct receptor sites on the VGSC protein, and binding to each of these sites has differential effects on channel kinetics. For example, TTX binds at site 1, irreversibly blocking the pore of the channel and preventing sodium from moving through the channel. Brevotoxin binds to site 5, enhancing activation and preventing inactivation of the channel. The binding of delta conotoxin to site 6 slows channel inactivation (Catterall et al. 2007).

Ample knowledge is also available for synthetic pyrethroid insecticides which bind to the sodium channel α subunit altering the normal gating kinetics of VGSC. Initial studies attempting to label the specific binding site of pyrethroids were unsuccessful due to the extreme lipophilicity and the modest potency of pyrethroid radioligands. The subsequent development of more potent radioligands demonstrated high affinity saturable binding to sodium channels in the brain. However, the high lipophilicity of pyrethroids still limited the sensitivity of the assay and obscured the identification of the single binding site responsible for pyrethroid action (Soderlund et al., 2002; Trainer et al., 1997). Despite these limitations, there remains overwhelming evidence that binding of pyrethroids to VGSC alters sodium channel kinetics. Mutations in the VGSC in insects alter gating kinetics by decreasing the sensitivity of the channel to pyrethroids, and provide resistance to their toxicity (Vais et al., 2000; 2001). In addition, the effects of pyrethroids are stereospecific where some isomers can interact with and modify channel function while other isomers are unable to bind and have no effect on channel kinetics (Soderlund 1985; Brown et al., 1988; Narahashi 1982). These properties of this class of insecticides on VGSCs are well established in the literature and have been extensively reviewed (by Soderlund et al. 2002).

Dose and temporal concordance

Dose-dependent actions of pyrethroids on VGSC kinetics are well documented in the peer-reviewed literature (see Song and Narahashi, 1996, Tabarean and Narahashi, 1998). On the other hand, temporal concordance for this KER is difficult to measure because of the rapidity (msec) with which chemical binding to the VGSC changes the conformation of the channel and its gating kinetics. However, from the detailed biological understanding of the KER it is clear that binding needs to precede the change in conformation

and gating kinetics. In addition, there is clear evidence that the binding to VGSC by some pyrethroids is dependent on particular states of the channel (e.g. open, closed, activated, deactivated). In such cases, modification of the channel kinetics is “use-dependent”, i.e., activation is increased with subsequent stimuli that result in channel opening (Wu et al., 2021; Tabarean and Narahashi, 2001). This characteristic of use-dependence is strong evidence that binding to the channel affects its gating properties.

Uncertainties and Inconsistencies

The fact that binding of chemicals to VGSCs results in altered sodium channel gate kinetics is well accepted and supported by abundant evidence. However, some minor uncertainties can be detected as reported below. Uncertainties in the overall knowledge remain; complete characterization of interactions of chemicals with all α isoforms of the channel, especially in mammals, as well as different subunit combinations have not been conducted, and differences likely exist based on different α - α and α - α / β subunit combinations. This is especially true for those channels that might be expressed during development, as the ontogeny of sodium channels is a complex process. Since brain development in both humans and rodents extends from early gestation well into the postnatal period it is not possible to state with certainty which isoform of the sodium channel’s α subunits is preferentially affected.

Quantitative Understanding of the Linkage

There are currently no quantitative models that predict the relationship between these KEs.

Response-response Relationship

There are currently no quantitative models that predict the relationship between these KEs. However, it is possible to compute the population of VGSC that are modified/affected by pyrethroid binding, and it has been estimated that less than 1% of the VGSC population (Narahashi et al., 1998) needs to be bound by pyrethroid to disrupt excitability in the neuron (KER2).

Chemicals may bind to VGSCs at various sites leading to different types of changes in the VGSC gate kinetics, and these changes also depend on the affinity of the chemicals to the binding sites (see section above, on KER description). Moreover, there are 9 different types of VGSCs including a complex ontology for the subunits. This complexity currently impedes the characterization of quantitative understanding.

Time-scale

The KER is active within milli-seconds and the upstream event occurs before the downstream event.

Known modulating factors

Species differences are demonstrated for orthologous channels with a high degree of amino acid sequence conservation, which differ in both their functional properties and their sensitivities to pyrethroid insecticides, e.g. with human $\text{Na}_v1.3$ channels being not only less sensitive than the rat $\text{Na}_v1.3$ channels but also less sensitive than rat $\text{Na}_v1.2$ channels (Sun et al., 2009)

Known Feedforward/Feedback loops influencing this KER

There are currently no known Feedforward/Feedback loops influencing this KER . Blank

Domain of Applicability

Most of the evidence for this key event comes from work in rodent species (i.e., rat, mouse) and in vitro human test systems Blank

References

Brown GB, Gaupp JE, Olsen RW. Pyrethroid insecticides: stereospecific allosteric interaction with the batrachotoxinin-A benzoate binding site of mammalian voltage-sensitive sodium channels. *Mol Pharmacol*. 1988 Jul;34(1):54-9. PMID: 2455860

Catterall WA, Cestèle S, Yarov-Yarovoy V, Frank HY, Konoki K and Scheuer T, 2007. Voltage-gated ion channels and gating modifier toxins. *Toxicon*, 49(2), 124–141. doi: 10.1016/j.toxicon.2006.09.022

Eijkelkamp N, Linley JE, Baker MD, Minett MS, Cregg R, Werdehausen R, Rugiero F, Wood JN. Neurological perspectives on voltage-gated sodium channels. *Brain*. 2012 Sep;135(Pt 9):2585-612.

Meyer DA, Carter JM, Johnstone AF and Shafer TJ, 2008. Pyrethroid modulation of spontaneous neuronal excitability and neurotransmission in hippocampal neurons in culture. *Neurotoxicology*, 29(2), 213–225. doi: 10.1016/j.neuro.2007.11.005.

Narahashi T, Aistrup GL, Lindstrom JM, Marszalec W, Nagata K, Wang F, Yeh JZ. Ion channel modulation as the basis for general anesthesia. *Toxicol Lett*. 1998 Nov 23;100-101:185-91. doi: 10.1016/s0378-4274(98)00184-2. PMID: 10049141.

Narahashi T. Cellular and molecular mechanisms of action of insecticides: neurophysiological approach. *Neurobehav Toxicol Teratol*. 1982 Nov-Dec;4(6):753-8.

O'Reilly AO, Khambay BP, Williamson MS, Field LM, Wallace BA and Davies TG, 2006. Modelling insecticide-binding sites in the voltage-gated sodium channel. *Biochemical Journal*, 396(2), 255–263.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, ... and Weiner ML, 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology*, 171(1), 3–59. [https://doi.org/10.1016/S0300-483X\(01\)00569-8](https://doi.org/10.1016/S0300-483X(01)00569-8)

Soderlund DM. Neurotoxicology. Pyrethroid-receptor interactions: stereospecific binding and effects on sodium channels in mouse brain preparations. 1985 Summer;6(2):35-46. PMID: 2410831

Song JH, Narahashi T. Modulation of sodium channels of rat cerebellar Purkinje neurons by the pyrethroid tetramethrin. *J Pharmacol Exp Ther*. 1996 Apr;277(1):445-53. PMID: 8613953

Sun XQ, Xu C, Leclerc P, Benoît G, Giuliano F, Droupy S. Spinal neurons involved in the control of the seminal vesicles: a transsynaptic labeling study using pseudorabies virus in rats. *Neuroscience*. 2009 Jan 23;158(2):786-97.

Tabarean IV, Narahashi T. Kinetics of modulation of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels by tetramethrin and deltamethrin. *J Pharmacol Exp Ther*. 2001 Dec;299(3):988-97. PMID: 11714887

Tabarean IV, Narahashi T. Potent modulation of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels by the type II pyrethroid deltamethrin. *J Pharmacol Exp Ther*. 1998 Mar;284(3):958-65.

Trainer VL, McPhee JC, Boutelet-Bochan H, Baker C, Scheuer T, Babin D, ... and Catterall WA, 1997. High affinity binding of pyrethroids to the α subunit of brain sodium channels. *Molecular Pharmacology*, 51(4), 651–657

Vais H, Atkinson S, Eldursi N, Devonshire AL, Williamson MS, Usherwood PN. A single amino acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides. *FEBS Lett*. 2000 Mar 24;470(2):135-8.

Vais H, Williamson MS, Devonshire AL, Usherwood PN. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. Pest Manag Sci. 2001 Oct;57(10):877-88.

Wu G, Li Q, Liu X, Li-Byarlay H, He B. Pestic Biochem Physiol. Differential state-dependent effects of deltamethrin and tefluthrin on sodium channels in central neurons of *Helicoverpa armigera*. 2021 Jun;175:104836.

Abbreviations

AOPs	Adverse outcome pathways
DNT	Developmental Neurotoxicity
DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
KE	Key Event
KERs	Key event relationships
MIE	Molecular initiating event
TTX	Tetrodotoxin
VGSCs	voltage-gated sodium channel

KER2: KER2625 Altered kinetics of sodium channel leads to Disruption in action potential generation

Key Event Relationship Title

Upstream event: Altered kinetic of sodium channel.

Downstream event: Disruption in action potential generation.

Key Event Relationship Overview

AOPs Referencing Relationship

Key Event Relationship Biological Domain of Applicability

Taxonomic Applicability:

Term	Scientific Term	Evidence
Vertebrates	Vertebrates	High
Invertebrates	Invertebrates	High

Sex Applicability

Sex	Evidence
Male	HIGH
Female	HIGH

Life Stage Applicability

Term	Evidence
all life stages, starting from foetal stage	HIGH

Key Event Relationship Description

Modification of VGSC kinetics may be represented by an alteration in the channel opening or closing. Some modifications, such as blocking by TTX, directly prevent the generation of an action potential. Other VGSC kinetic kinetics may shift the membrane potential required to trigger an action potential. Modification of VGSC kinetics may also be represented by slowing down the activation and inactivation of the channel. This slowing of the timeline increases the channel opening time producing a population of channels that remain open when unmodified channels have closed. A direct consequence of persistent channel opening is depolarization of the membrane to action potential threshold and the induction of repetitive firing of the cell.

However, if the channel is held open for a sufficiently long period, the membrane potential eventually becomes depolarized to the point that generation of action potentials is not possible (depolarization-dependent block). Thus, the effects of disruption VGSC kinetics on the action potential are qualitatively different based on the time the channel remains open and this can be measured electrophysiologically. A limited chemically-induced increase in channel opening will lead to repetitive firing while a prolonged opening blocks action potential generation (Shafer et al., 2005).

Biological Plausibility

The biological plausibility of KER2 (*Altered kinetics of sodium channel leads to Disruption in action potential generation*) is strong. The rising phase of an action potential is caused by the opening of voltage-gated sodium channels. These ion channels are activated once the cell's membrane potential reaches a threshold and open immediately. The electrochemical gradients drive sodium into the cell causing a strong and abrupt depolarization characteristic of an action potential. The falling phase of the action potential is caused by the inactivation of the VGSCs stopping further sodium influx, and the opening of voltage-gated potassium channels. As K⁺ concentrations inside the cell are very high, channels open and the current flow out serves to restore the membrane potential toward its resting state. However, the efflux of K⁺ ions is large leading to a hyperpolarisation (undershoot phase) of the membrane potential. Ultimately the voltage-gated K⁺ channels close and the membrane potential returns to its resting state. This is very well-established textbook knowledge. While it is well accepted that various combinations of channel types in a cell can give rise to differences in the shape and time course of the action potential, the underlying biological principles and relationships between VGSC and action potentials are maintained. Expression of VGSCs is spatially and temporally dependent and have differential expression during CNS development. ~~It is clear that as~~ As in the adult, binding to VGSC isoforms will also disrupt the channel gating kinetics and action potential generation in the developing brain (see reviews by Shafer et al., 2005; Soderlund et al., 2002).

Empirical Evidence

The empirical evidence of KER2 is strong. As described in KE2, natural toxins like TTX bind to VGSC and block all electrical activity including action potentials. The relationship of VGSC and action potential generation has been widely demonstrated with a variety of other stressors (e.g., local anesthetics, anticonvulsants and other pharmacological agents (Hwang et al., 2020; Lee et al., 2015). A large body of literature on pyrethroids insecticides has confirmed their ability to alter action potential firing in both insect and mammalian peripheral and central *in vitro* and *in vivo* preparations. These studies have been extensively reviewed (Soderlund et al., 2002; Narahashi et al., 1998; Bloomquist, 1996).

Dose and temporal concordance

Although it is well established that altered kinetics of VGSC lead to disruption of action potentials, due to the “all or none” firing characteristics of neuronal action potentials, it is difficult to demonstrate dose-concordance for this specific KER. However, there are examples in the literature showing dose concordance between concentration of pyrethroid insecticides and action potential generation. For deltamethrin and permethrin, changes in VGSC kinetics and disruption of the action potential are reported *in vitro* at concentration between 0.01 to 1 mM, in hippocampal or neocortical neurons from postnatal day 2–4 pups (Meyer et al., 2008; Cao et al., 2011). Similarly, Song and Narahashi (1995; 1996) demonstrated dose concordance for tetramethrin. As described here and in KER1, there is substantial evidence of dose-related effects of pyrethroid insecticides.

| Similar to Like the temporal concordance between binding of compound to the VGSC and altered kinetics (KER1), alteration of VGSC kinetics and membrane excitability/action potential generation also occur very quickly. Thus, temporal concordance is difficult to demonstrate directly using experimental approaches. As mentioned, pyrethroid effects have been described as “use-dependent”. As such, increased modification of the channel with repeated depolarizations results in further disruption of action potentials.

Uncertainties and Inconsistencies

Evidence supporting this KER is derived nearly entirely from *in vitro* experiments, as it is not possible to measure directly sodium channel function *in vivo*, only proxies of it. However, *in vivo* recordings of action potentials demonstrate repeated firing in both mammalian and non-mammalian species, supporting that the KER relationship exists across species and in intact nervous systems. Additional uncertainty exists due to the diversity of different sodium channel subunits and understanding their role in the action potential. Thus, the exact compositions of sensitive channels are not characterized. With respect to temporal relationships, different pyrethroid compounds exhibit differing levels of use dependence (Soderlund, 2010), which can be influenced by channel type.

However, the level of evidence supporting this KER in the peer-reviewed literature is abundant and the confidence in this KER is high.

Quantitative Understanding of the Linkage

Generation of action potentials and the roles of different ion channels in action potential generation and propagation are well understood, and described by the Hodgkin-Huxley model, so theoretically, a quantitative model could be constructed that incorporates alterations in VGSC kinetics and links to action potential generation. It has been estimated that an increased in opening time of a small percentage of VGSCs (< 1% of the VGSCs in a neuron) is all that is required to trigger repetitive firing of that neuron, and an accelerated cycling of the naïve VGSC to cycle through their resting/open/inactivation stages (Narahashi, 1996). Computational models of VGSC conductance and action potential generation have been published (Santha-Kumar et al., 2005). These models exemplify both dose and temporal concordance between these two KEs.

Response-response Relationship

The relationship between alteration of VGSC kinetics and action potential generation has been modeled in neuroblastoma cells for tetramethrin (Mohan et al., 2006; Molnar and Hickman, 2014). However, the extent to which this model has been extended to other pyrethroids is not clear.

Time-scale

The KE channel opening lasts micro-seconds and modification by compounds occurs quickly, but in the case of state dependence, can be exacerbated with repeated depolarization. Action potential typically last less than a millisecond under normal biological conditions. Modification of the VGSC by pyrethroids can result in repeated firing of action potentials that occur for hundreds of milliseconds (e.g., Song and Narahashi, 1996). Thus, KER happens within milliseconds to microsecond time-scale.

Known modulating factors

As noted above, the composition of different VGSC channel subunits, as well as compositions of voltage-gated potassium and calcium channels in the cell, can influence the overall shape and timing of the action potential. This includes changes that might be the result of developmentally specific expression of channels and subunits.

Known Feedforward/Feedback loops influencing this KER

As described above, the state-dependent interaction of pyrethroids can result in exacerbation of effects with repeated depolarizations. When VGSC inactivation occurs at short intervals, action potentials are fired repetitively. Such is the case for permethrin and other Type I pyrethroids. By contrast, pyrethroids (type II) prolong VGSC inactivation for a longer [period of time](#), depolarizing the membrane potential to the point that action potentials can no longer be generated - depolarization-dependent block (Shafer et al., 2005).

Domain of Applicability

The relationship between activity of VGSC and action potential generation is well described in the literature and highly conserved from low-level phyla (e.g. planarians) to humans, is present in both sexes and throughout development (Smith and Walsh, 2020).

References

Bloomquist JR. Ion channels as targets for insecticides. *Annu Rev Entomol.* 1996; 41:163-90.

Cao Z, Shafer TJ and Murray TF, 2011. Mechanisms of pyrethroid insecticide-induced stimulation of calcium influx in neocortical neurons, *Journal of Pharmacology and Experimental Therapeutics*, 336 (1), 197–205. American Society for Pharmacology and Experimental Therapeutics. doi: <https://doi.org/10.1124/jpet.110.171850>

Hwang, K.S., Kan, H., Kim, S.S., Chae, J.S., Yang, J.Y., Shin, D.S., Ahn, S.H., Ahn, J.H., Cho, J.H., Jang, I.S., Shin, J., Joo, J., Kim, C.H., Bae, M.A. (2020) Efficacy and pharmacokinetics evaluation of 4-(2-chloro-4-fluorobenzyl)-3-(2-thienyl)-1,2,4-oxadiazol-5(4H)-one (GM-90432) as an anti-seizure agent. *Neurochemistry international*. 141:104870.

Lee KH, Lee H, Yang CH, Ko JS, Park CH, Woo RS, Kim JY, Sun W, Kim JH, Ho WK, Lee SH. Bidirectional Signaling of Neuregulin-2 Mediates Formation of GABAergic Synapses and Maturation of Glutamatergic Synapses in Newborn Granule Cells of Postnatal Hippocampus. *J Neurosci.* 2015 Dec 16;35(50):16479-93.

Meyer DA, Carter JM, Johnstone AF and Shafer TJ, 2008. Pyrethroid modulation of spontaneous neuronal excitability and neurotransmission in hippocampal neurons in culture. *Neurotoxicology*, 29(2), 213–225. doi: 10.1016/j.neuro.2007.11.005.

Mohan DK, Molnar P, Hickman J. Toxin detection based on action potential shape analysis using a realistic mathematical model of differentiated NG108-15 cells. *J.Biosens Bioelectron.* 2006 Mar 15;21(9):1804-11. doi: 10.1016/j.bios.2005.09.008. Epub 2006 Feb 3. PMID: 16460924 Morgan and Soltesz, 2008

Molnar P, Hickman J Modeling of action potential generation in NG108-15 cells. *J.Methods Mol Biol.* 2014;1183:253-61. doi: 10.1007/978-1-4939-1096-0_16. PMID: 25023314

Narahashi T, 1996. Neuronal ion channels as the target sites of insecticides. *Pharmacology and Toxicology*, 79(1), 1–14.

Narahashi T, Aistrup GL, Lindstrom JM, Marszalec W, Nagata K, Wang F, Yeh JZ. Ion channel modulation as the basis for general anesthesia. *Toxicol Lett.* 1998 Nov 23;100-101:185-91. doi: 10.1016/s0378-4274(98)00184-2. PMID: 10049141.

Santhakumar V, Aradi I, Soltesz I. (2005). Role of mossy fiber sprouting and mossy cell loss in hyperexcitability: a network model of the dentate gyrus incorporating cell types and axonal topography. *Journal of neurophysiology.* 93

Shafer TJ, Meyer DA and Crofton KM, 2005. Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environmental Health Perspectives*, 113(2), 123–136. <https://doi.org/10.1289/ehp.7254>

Smith RS, Walsh CA. Ion Channel Functions in Early Brain Development. *Trends Neurosci.* 2020 Feb;43(2):103-114. doi: 10.1016/j.tins.2019.12.004. Epub 2020 Jan 17. PMID: 31959360; PMCID: PMC7092371.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, and Weiner ML, 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology*, 171(1), 3–59.

Soderlund DM. State-Dependent Modification of Voltage-Gated Sodium Channels by Pyrethroids. *Pestic Biochem Physiol.* 2010 Jun 1;97(2):78-86. doi: 10.1016/j.pestbp.2009.06.010. PMID: 20652092

Song JH, Narahashi T. Modulation of sodium channels of rat cerebellar Purkinje neurons by the pyrethroid tetramethrin. *J Pharmacol Exp Ther.* 1996 Apr;277(1):445-53. PMID: 8613953

Song JH, Narahashi T. Selective block of tetramethrin-modified sodium channels by (+/-)-alpha-tocopherol (vitamin E). *J Pharmacol Exp Ther.* 1995 Dec;275(3):1402-11.

Glossary [and/or] Abbreviations

AOPs	Adverse outcome pathways
CNS	Central Nervous System
KE	Key Event
KERs	Key event relationships
TTX	Tetrodotoxin
VGSCs	voltage-gated sodium channel

| **KER3: KER3242: Disruption of action potential leads to altered neurotransmission during development.**

Key Event Relationship Title

Upstream event: Disruption of action potential

Downstream event: Altered neurotransmission during development

Key Event Relationship Overview

AOPs Referencing Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence
Vertebrates	Vertebrates	HIGH
Invertebrates	Invertebrates	HIGH

Sex Applicability

Sex	Evidence
Male	HIGH
Female	HIGH

Life Stage Applicability

Term	Evidence
All life stages starting from foetal stage	HIGH

Key Event Relationship Description

Stimulation of neurons by neurotransmitters or sensory input activates the opening of different ion channels and permits current flow across the membrane. As ion currents move across the membrane, the membrane potential is changed. Depending on the ion channel, this change in membrane potential can be either excitatory to depolarize, or inhibitory to hyperpolarize the cell. Neurons integrate the barrage of both excitatory and inhibitory signals they receive. When this integration leads to a net sum depolarization, voltage gated sodium channels open and an action potential is triggered. The action potential through a series of successive openings of additional VGSCs, allows the transmission of the electrical impulses to move along the length of the axon to the nerve terminal. The synapse describes the location where the presynaptic nerve terminal meets the postsynaptic cell. The postsynaptic cell can be another neuron, muscle or gland. At the synapse the electrical signal at the presynaptic terminal is transduced to a chemical signal to span the spatial gap and communicate information from one cell to the next. On arrival of the depolarizing action potential (AP) at the presynaptic terminal, voltage gated calcium channels are activated and vesicles containing chemical neurotransmitters are released into the synaptic cleft – the space between the neurons. The frequency and duration of the action potentials determines how many neurotransmitter vesicles are released. The neurotransmitters act on the postsynaptic cell by interaction with neurotransmitter-specific receptors that depolarize or hyperpolarize the membrane of the receiving cell. This transduction of electrical to chemical and back again to electrical

signaling across neurons is the basis of neurotransmission. This sequence of events is portrayed in Figure 4.

Sequence of Events From Action Potential Generation to Synaptic Transmission

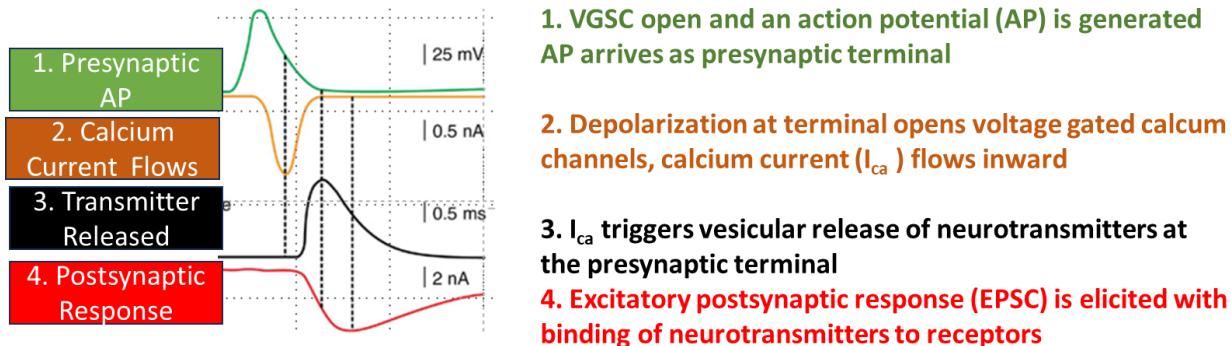


Figure 4. Sequence of Events from action potential generation to synaptic transmission. Self produced by EFSA WG.

It is well established that neurotransmission can be disrupted through several different mechanisms, including disruptions of ion channels, release machinery, post-synaptic response and disruption of neurotransmitter re-uptake or degradation (Atchison, 1988; Vester and Caudle, 2016). It is also well accepted that neurotransmission occurs in the mature and developing brain and can be similarly disrupted by the same mechanisms.

Evidence Supporting this KER

The evidence supporting this KER a well-established tenant of neurobiology (Foundations of Neuroscience by Casey Henley; <https://openbooks.lib.msu.edu/neuroscience/chapter/drug-and-toxin-effects/> ; more detailed in Cellular and Molecular Neurophysiology There is abundant evidence that disruption of action potentials leads to altered neurotransmission by drugs and environmental agents, including VGSC blockers (Meng et al., 2016; Shafer et al., 2008; Hossain et al., 2008; for review, see Soderlund et al., 2002). There are also numerous examples of peer-reviewed studies demonstrating alterations in action potential activity leading to altered neurotransmission during development (Čechová and Šlamberová, 2021; Latchney et al., 2021).

Biological Plausibility

The process of disruption of action potentials leading to changes in neurotransmission represents a very well-established principle of neurobiology that is widely described in the published literature and basic neuroscience textbooks. This process is the basis of routine neurophysiological studies investigating the development, function and disturbance of neuronal networks. It is not only biologically plausible that alterations in action potential shape, duration and patterns could lead to altered neurotransmission, but also that this occurs in adult and developing nervous systems.

Empirical Evidence

The empirical evidence of KER3 is strong. There is abundant empirical evidence in the published literature supporting the basic biology underlying this KER. A variety of insults ~~including chemical insults~~ can alter action potential generation and impair synaptic transmission (e.g. Seabrook et al., 1989; Joy et al., 1990; Hong et al., 1986; Gilbert et al., 1989. Eells and Dubocovich, 1988; Hossain et al., 2008; Shafer et al., 2008). These data have been generated in a wide variety of different models from insects to mammalian models, including embryonic neurons and adult neuronal preparations. For a more detailed explanation, and examples of chemicals and mechanisms leading to altered neurotransmission, the readers are referred to <https://openbooks.lib.msu.edu/neuroscience/chapter/drug-and-toxin-effects/>

Dose and temporal concordance

Because of the challenges of measuring this KER, dose concordance is not well established. However, there is no indication of discontinuity between dose levels that alter action potential activity and synaptic transmission. In addition, dose-concordance is observed at both earlier and later KERs, indicating that it likely is maintained for this KER.

Action potential stimulation of neurotransmission occurs in a very rapid millisecond time scale. There is strong evidence for temporal concordance between alterations in action potential activity and changes in neurotransmission. The sequence of events from presynaptic action potential generation and postsynaptic response as depicted above in Figure 4, clearly demonstrates the time concordance between these two KEs.

Uncertainties and Inconsistencies

The biological processes that regulate the generation and propagation of action potential and neuronal transmission are very well known and changes in this KER are well documented for chemical insults. This KER is supported by both *in vitro* and *in vivo* data.

The literature directly demonstrating the relationship between action potential generation and neurotransmission *during development* is less robust. However, given the

fundamental properties of neurotransmission that exist in both mature and developing nervous system and the extensive literature of chemical stressors derived from a wide variety of preparations of varying ages, this uncertainty is small.

Quantitative Understanding of the Linkage

Currently, quantitative models for this KER were not found in the peer-reviewed literature.

Response-response Relationship

The overall relationship between action potential firing leading to release of neurotransmitter release and a response in the post-synaptic cell is well established in neurobiology. One simple example is the firing of a motor neuron, leading to release of acetylcholine, followed by muscle contraction. The sequence of events from presynaptic action potential generation and postsynaptic response as depicted above in Figure 1, clearly demonstrates the response-response concordance between these two KEs. The precise form of the response-response relationships in terms of the either excitation or inhibition and strength of that effect is dependent on the neuron type and its location and function within the nervous system.

Time-scale

The KER is active within milli-seconds and the upstream event occurs before the downstream events (see Figure 4 above)

Known modulating factors

The description of the KER provided here is a generic description, but the basic biology described is maintained across species, developmental stage, brain regions and sex. There are a number of factors that can modulate this relationship, including, but not limited to, temperature; region/pathway/neuronal subtype, type of synaptic structure, age of the animal and preceding activity at that synapse.

Known Feedforward/Feedback loops influencing this KER

There are currently no known Feedforward/Feedback loops influencing this KER .

Domain of Applicability

Male, females, all life stages, starting from foetal stage (Smith and Walsh 2020).

References

Atchison WD. Effects of neurotoxicants on synaptic transmission: lessons learned from electrophysiological studies. *Neurotoxicol Teratol*. 1988 Sep-Oct;10(5):393-416. doi: 10.1016/0892-0362(88)90001-3. PMID: 2854607

Čechová B, Šlamberová R. Methamphetamine, neurotransmitters and neurodevelopment. *Physiol Res*. 2021 Dec 31;70(S3):S301-S315. doi: 10.33549/physiolres.934821. PMID: 35099249; PMCID: PMC8884400.

Cellular and Molecular Neurophysiology book, 4th edition. 2015. Soderlund et al., 2002

Eells JT, Dubocovich ML. Pyrethroid insecticides evoke neurotransmitter release from rabbit striatal slices. *J Pharmacol Exp Ther*. 1988 Aug;246(2):514-21. PMID: 3404444.

Foundations of Neuroscience by Casey Henley; <https://openbooks.lib.msu.edu/neuroscience/chapter/drug-and-toxin-effects/>

Gilbert ME, Mack CM, Crofton KM. Pyrethroids and enhanced inhibition in the hippocampus of the rat. *Brain Res*. 1989 Jan 16;477(1-2):314-21. doi: 10.1016/0006-8993(89)91420-0. PMID: 2702491.

Hong JS, Herr DW, Hudson PM, Tilson HA. Neurochemical effects of DDT in rat brain in vivo. *Arch Toxicol Suppl*. 1986;9:14-26. doi: 10.1007/978-3-642-71248-7_2. PMID: 2434059.

Hossain MM, Suzuki T, Unno T, Komori S, Kobayashi H. Differential presynaptic actions of pyrethroid insecticides on glutamatergic and GABAergic neurons in the hippocampus. *Toxicology*. 2008 Jan 14;243(1-2):155-63. doi: 10.1016/j.tox.2007.10.003. Epub 2007 Oct 10. PMID: 18023957.

Hossain, M.M.; Suzuki, T.; Unno, T.; Komori, S.; Kobayashi, H. Differential presynaptic actions of pyrethroid insecticides on glutamatergic and gabaergic neurons in the hippocampus. *Toxicology* 2008, 243, 155–163.

Joy RM, Lister T, Ray DE, Seville MP. Characteristics of the prolonged inhibition produced by a range of pyrethroids in the rat hippocampus. *Toxicol Appl Pharmacol*. 1990 May;103(3):528-38. doi: 10.1016/0041-008x(90)90325-o. PMID: 2339424.

Latchney SE, Majewska AK. Persistent organic pollutants at the synapse: Shared phenotypes and converging mechanisms of developmental neurotoxicity. *Dev Neurobiol.* 2021 Jul;81(5):623-652. doi: 10.1002/dneu.22825. Epub 2021 May 2. PMID: 33851516; PMCID: PMC8364477.

Meng L, Meyer PF, Leary ML, Mohammed YF, Ferber SD, Lin JW. *Neurosci Lett.* 2016. Effects of Deltamethrin on crayfish motor axon activity and neuromuscular transmission. Mar 23;617:32-8. doi: 10.1016/j.neulet.2016.01.061. Epub 2016 Feb 6. PMID: 26861201

Seabrook GR, Duce IR, Irving SN. Spontaneous and evoked quantal neurotransmitter release at the neuromuscular junction of the larval housefly, *Musca domestica*. *Pflugers Arch.* 1989 May;414(1):44-51. doi: 10.1007/BF00585625. PMID: 2566966.

Shafer TJ, Rijal SO, Gross GW. Complete inhibition of spontaneous activity in neuronal networks in vitro by deltamethrin and permethrin. *Neurotoxicology.* 2008 Mar;29(2):203-12. doi: 10.1016/j.neuro.2008.01.002. Epub 2008 Jan 19. PMID: 18304643.

Shafer TJ, Rijal SO, Gross GW. *Neurotoxicology.* 2008. Complete inhibition of spontaneous activity in neuronal networks in vitro by deltamethrin and permethrin. Mar;29(2):203-12. doi: 10.1016/j.neuro.2008.01.002. Epub 2008 Jan 19. PMID: 18304643

Smith RS, Walsh CA. Ion Channel Functions in Early Brain Development. *Trends Neurosci.* 2020 Feb;43(2):103-114. doi: 10.1016/j.tins.2019.12.004. Epub 2020 Jan 17. PMID: 31959360; PMCID: PMC7092371.

Soderlund., 2002. Cellular and Molecular Neurophysiology book, 4th edition. 2015.

Vester A, Caudle MW. The Synapse as a Central Target for Neurodevelopmental Susceptibility to Pesticides Toxics. 2016 Aug 26;4(3):18.

Glossary [and/or] Abbreviations

AOPs	Adverse outcome pathways
AP	Action potential
CNS	Central Nervous System
EPSC	Excitatory synaptic currents
KE	Key Event
KERs	Key event relationships
TTX	Tetrodotoxin
VGSCs	voltage-gated sodium channel

KER4: KER3243: Altered neurotransmission during development leads to altered hippocampal anatomy

Key Event Relationship Title

Upstream event: **Altered neurotransmission during development**

Downstream event: **Altered hippocampal anatomy**

Key Event Relationship Overview

AOPs Referencing Relationship

Key Event Relationship Biological Domain of Applicability

This KER is supported from rodent models, in which hippocampal brain slices have been studied *ex vivo*. Despite the hippocampus is structurally quite different among mammals, birds and reptiles, its function in spatial memory is highly conserved (Striedter, 2016). This suggests, with some uncertainty, that this KER is also applicable to multiple species.

Taxonomic Applicability:

Term	Scientific Term	Evidence
Vertebrates	Vertebrates	High
Invertebrates	Invertebrates	High

Term	Scientific Term	Evidence
Rat	<i>Rattus norvegicus</i>	Moderate
Mouse	<i>Mus musculus</i>	Moderate
Human	<i>Homo sapiens</i>	Not Specified

Sex Applicability:

Sex	Evidence
Male	High
Female	High

Life Stage Applicability

Term	Evidence
During development	high

Key Event Relationship Description

It is well established that neurons extend and retract their pre- and postsynaptic processes dependent on the level of neuronal activation (Andrae et al., 2014). These growth processes determine the basic shape of a neuron and its regions of afferent and efferent connections, processes critical during brain development. Neural systems encode information structurally via the wiring between neurons and this wiring is modulated by the electrical activity in both the developing and adult nervous systems. Establishment of synaptic connectivity begins as a diffuse process that is refined in an activity-dependent manner during development (Pan and Monje, 2020). Disruption of the formation of precise neural circuits during the prenatal and perinatal stages of brain development may underlie neurodevelopmental disorders.

At birth, an infant's brain contains more neurons than present in the adult. As the child grows, experiences strengthen circuits that prove more relevant and weaken others. This process of overgrowth followed by selective activity-dependent elimination is key to forming an adaptive brain, with waves of neuronal cell death and dramatic reduction in connecting axonal fibers occurring during development (Anosike et al., 2023). The process whereby a subset of synapses is removed, while others are maintained is called synaptic pruning. It is a fundamental property of the mammalian CNS, it occurs in response to changes in neural activity, and it is most prominent in the developing nervous system (Faust et al., 2021). The lack of activity at the majority of most synapses on a neuron can lead to the eventual death of that cell. As such, interference with electrical signaling during development can certainly influence connectivity of the developing brain.

The activity-dependent processes alter both the structure of the axonal bouton of presynaptic and the dendritic spine of the postsynaptic neuron. Spines can change in shape, volume, density, and location. Overall activity levels can increase or decrease spine number, dendritic branches can be expanded or eliminated, axons removed or redirected to novel destinations based on the level of activity with the synaptic network. Denervation inducing a total lack of neuronal activity can induce axonal growth and expansion to other areas.

Spontaneous neurotransmitter release plays an important role in shaping neuronal morphology as well as modulating the properties of newly forming synaptic connections in the brain (Andreae and Burrone, 2018). Excessive or insufficient neurotransmission during critical windows of development can affect the complexity of the connectivity within pre- and post-synaptic neurons, leading to altered synaptic density and connectivity. The delicate balance between excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission shapes brain circuitry, and when perturbed, it can lead to abnormal network activity (Cherubini et al., 2021). This has been widely studied in the hippocampus.

There are two types of structural remodelling, one ~~occurring on~~occurs fast (minutes to hours), the other on a more protracted (hours, days, weeks) timescale. Activity-dependent remodelling can occur at ultrastructural, network, and regional levels, and in the developing nervous system as well as the mature brain (see review by Fauth and Tetzlaff, 2016). Changes in neuronal architecture driven by activity are known to occur in all brain areas studied to date including the hippocampus.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of altered neurotransmission during the development and further impairment of hippocampal anatomy is strong. Extensive evidence supports the notion that disruption of neurotransmission during development can induce micro-structural morphological changes in the hippocampus. This can occur ~~due to the effect because~~ of various factors such as genetic mutations, brain damage, environmental toxins, and stress during vulnerable periods of brain development.

Impaired synaptic transmission may occur at pre- or postsynaptic level and involves disruption of the normal functioning of neurotransmitters, their receptors, or scaffolding proteins. The strength of the synaptic transmission can be modulated by the amount of neurotransmitter released, the number of receptors on the postsynaptic cell, and their sensitivity to the neurotransmitter due to alterations in the number and conductance of postsynaptic receptors (Graziane and Dong, 2022; Hestrin, 2015). In case of presynaptic dysfunction, either too much or too little neurotransmitter may be released into the synaptic cleft, whereas in postsynaptic dysfunction, the postsynaptic neuron may not respond adequately to that neurotransmitter. In both cases, the altered synaptic transmission may have pre- or postsynaptic morphological consequences, including e.g. number of docked vesicles at the nerve terminal, or the number, density and morphology of dendrite spines. These changes may affect the structure and function of neural circuits and may underlie behavioral deficits (Bonycastle et al., 2021).

Empirical Evidence

The evidence supporting this KER is considered moderate. Neurites of single cells in culture grow and retract depending on the level of neuronal activation (Cohan and Kater, 1986). Pharmacological block of action potentials by saxitoxin curtails synaptic transmission in PC12 and SH-SY5Y cell lines and inhibits neurite outgrowth (O'Neill et al., 2017). Electrical stimulation to activate synaptic transmission induces rapid input-specific changes in dendritic structure; however, these changes are reversed when

neurotransmission is blocked (Kirov and Harris, 1999). These phenomena have been demonstrated in developing hippocampal cultures, dissociated neuronal cultures, organotypic slices and in intact organisms. The number, volume, density, and shape of dendritic spines can all be altered with electrical stimulation. Spine growth is input specific, occurs only close to activated parts of the dendrite, and can be eliminated by blocking synaptic transmission at the postsynaptic receptor. Chronic blockade of neuronal activity leads to the reversible growth of dendritic spines in the hippocampus, while persistent activity-dependent changes in spine structure contributes to the development and refinement of neural circuitry (Maletic-Savatic et al., 1999; Kirov and Harris, 1999).

Cultured cortical neurons deprived of action potentials by an extended period of tetrodotoxin (TTX) treatment initially showed a marked increase in size and frequency of mEPSCs, indicating a rise in the postsynaptic response to glutamate. Morphologically, these neurons retracted their dendrites, lost dendritic spines, and eventually degenerated over a period of 1–2 weeks. Neuronal morphological deterioration was prevented by blockade of glutamatergic AMPA receptors (Fishbein and Segal, 2007). As such, the block of action potential generation and consequent neurotransmission impairment can lead to altered morphology by both direct and indirect means.

Both higher and lower levels of activity can drive structural change in positive and negative directions, at ultrastructural and macrostructural scales. For example, unrelated to neuronal damage, elevated levels of electrical activity accompanying epilepsy reduce spine number (Geineisman et al., 1990). Sensory deprivation leading to lower activity levels in neurons can increase the number of newly formed spines. Some examples include monocular deprivation in the mouse that eliminates electrical activity in visual cortex neurons in one hemisphere, doubles the number of newly formed spines in the binocular region of the same hemisphere (Hofer et al., 2009). Similarly, trimming the whiskers of rats to eliminate excitation of somatosensory neurons leads to an increased number of spines and an outgrowth of dendritic trees into the barrel field of the cortex (Vees et al., 1998). With a delay of several days, axons from the neighboring neurons, unaffected by the deprivation, grow toward the deprived region. These adjacent neurons, although unaffected by the deprivation, experience altered activity levels, triggering their axonal growth. In both visual and somatosensory models, structural plasticity is most pronounced during specific limited time windows in brain development.

In the hippocampus, electrical stimulation of afferents alters spine number and morphology of pyramidal and granule cell neurons *in vitro* and *in vivo* (Kirov et al., 2004; Kirov and Harris, 1999; Geineisman et al., 1990; Maletoc-Savatic et al., 1999) and increases neurogenesis in the adult dentate gyrus (Chun et al., 2006; 2009; Gilbert et al., 2020).

Activity-dependent structural changes in connectivity have been amply documented in adult networks and in the developing brain. It is widely accepted that activity-dependent morphological growth and restructuring is paramount in development. Specific patterns

of change may be different in the mature versus the developing nervous system, but that activity is the trigger of structural change is not in doubt.

Dose and temporal concordance

Dose-response data is lacking for this KER. For future research, it is critical to generate data in which the upstream KE is modulated in a 'dose-response' manner to better support the causative relationship.

Essentiality

The evidence is clear that synapse formation, synapse pruning, and the establishment and fine tuning of neural circuits in the developing brain requires neurotransmission. As such, alterations in neurotransmission during development drive changes in post-synaptic structure in the hippocampus.

Uncertainties and Inconsistencies

Changes in connectivity have not been directly linked to electrical activity per se, but neuronal activity is essential to trigger complex molecular signaling cascades, which mediate to the corresponding structural changes. In many cases, calcium signaling is used as a surrogate measure of electrical activity at the synapse as postsynaptic calcium level is largely dictated by neuronal activity. However, the detailed relation between calcium, activity, and spine dynamics is more complex, as the calcium level is also regulated by other signals such as neurotrophins and adhesion molecules (Stoop and Poo, 1996; Bixby et al., 1994).

Quantitative Understanding of the Linkage

Several theoretical and computational models of structural plasticity exist and range from simple single neuron connections to complex neural networks. Both dynamics of dendritic spines on a brief timescale to longer timelines of structural connectivity have been described and reviewed by Fauth and Telzlaff (2016).

Response-response Relationship

The connection between activity and structural change is well documented and the nature of the structural alteration can be growth and stabilization or destabilization and elimination at the synaptic level. Elimination of entire neurons can occur in complete absence of activity, while at the same time, absence of activity can trigger growth of adjacent neurons to a denervated site.

Time-scale

Seconds to minutes, hours to days, days to weeks, mature and immature organisms

Known modulating factors

Although activity dependent alterations in synaptic structure occur in both males and females, hormones can modulate their extent, serving to stabilize connections in some cases, while destabilizing and eliminating connections in others. Other hormonal systems, notably glucocorticoids can modulate activity-dependent structural change.

Known Feedforward/Feedback loops influencing this KER

There are currently no known Feedforward/Feedback loops influencing this KER .

Domain of Applicability

Activity-dependent alterations in brain connectivity and synaptic structure occurs in males and females, in all mammals, at all life stages, and is especially prominent during development. Structural remodeling also occurs in the non-mammalian species.

References

Andreae LC, Burrone J. The role of neuronal activity and transmitter release on synapse formation. *Curr Opin Neurobiol.* 2014 Aug;27(100):47-52. doi: 10.1016/j.conb.2014.02.008

Andreae LC, Burrone J. The role of spontaneous neurotransmission in synapse and circuit development. *J Neurosci Res.* 2018 Mar;96(3):354-359. doi: 10.1002/jnr.24154

Anosike NL, Adejuwon JF, Emmanuel GE, Adebayo OS, Etti-Balogun H, Nathaniel JN, Omotosho OI, Aschner M, Ijomone OM. Necroptosis in the developing brain: role in neurodevelopmental disorders. *Metab Brain Dis.* 2023 Mar;38(3):831-837. doi: 10.1007/s11011-023-01203-9

Bixby JL, Grunwald GB, Bookman RJ. Ca²⁺ influx and neurite growth in response to purified N-cadherin and laminin. *J Cell Biol.* 1994 Dec;127(5):1461-75. doi: 10.1083/jcb.127.5.1461

Bonycastle K, Davenport EC, Cousin MA. Presynaptic dysfunction in neurodevelopmental disorders: Insights from the synaptic vesicle life cycle. *J. Neurochem.* 2021; 157: 179–207. <https://doi.org/10.1111/jnc.15035>

Cherubini E, Di Cristo G, Avoli M. Dysregulation of GABAergic Signaling in Neurodevelopmental Disorders: Targeting Cation-Chloride Co-transporters to Re-establish a Proper E/I Balance. *Front Cell Neurosci.* 2022 Jan 5;15:813441. doi: 10.3389/fncel.2021.813441

Chun SK, Sun W, Park JJ, Jung MW. Enhanced proliferation of progenitor cells following long-term potentiation induction in the rat dentate gyrus. *Neurobiol Learn Mem.* 2006 Nov;86(3):322-9. doi: 10.1016/j.nlm.2006.05.005. Epub 2006 Jul 7. PMID: 16824772.

Chun SK, Sun W, Jung MW. LTD induction suppresses LTP-induced hippocampal adult neurogenesis. *Neuroreport.* 2009 Sep 23;20(14):1279-83. doi: 0.1097/WNR.0b013e3283303794. PMID: 1963358

Cohan CS, Kater SB, 1986. Suppression of neurite elongation and growth cone motility by electrical activity. *Science*, 27;232(4758):1638-40. doi: 10.1126/science.3715470

Faust TE, Gunner G, Schafer DP, 2021. Mechanisms governing activity-dependent synaptic pruning in the developing mammalian CNS. *Nat Rev Neurosci* 22, 657–673. <https://doi.org/10.1038/s41583-021-00507-y>

Fauth M, Tetzlaff C. Opposing Effects of Neuronal Activity on Structural Plasticity. *Front Neuroanat.* 2016 Jun 28;10:75. doi: 10.3389/fnana.2016.00075

Fishbein I, Segal M. Miniature synaptic currents become neurotoxic to chronically silenced neurons. *Cereb Cortex.* 2007 Jun;17(6):1292-306. doi: 10.1093/cercor/bhl037

Genisman Y, Morrell F, deToledo-Morrell L. Increase in the relative proportion of perforated axospinous synapses following hippocampal kindling is specific for the synaptic field of stimulated axons. *Brain Res.* 1990 Jan 22;507(2):325-31. doi: 10.1016/0006-8993(90)90291-i

Gilbert J, O'Connor M, Templet S, Moghaddam M, Di Via Ioschpe A, Sinclair A, Zhu LQ, Xu W, Man HY. NEXMIF/KIDLIA Knock-out Mouse Demonstrates Autism-Like Behaviors, Memory Deficits, and Impairments in Synapse Formation and Function. *J Neurosci.* 2020 Jan 2;40(1):237-254. doi: 10.1523/JNEUROSCI.0222-19.2019

Graziane N, Dong Y. (2022). Isolation of Synaptic Current. In: Graziane N, Dong Y (eds) *Electrophysiological analysis of synaptic transmission. Neuromethods*, vol 187. Humana, New York, NY, 2022, pp 101–110 (https://doi.org/10.1007/978-1-0716-2589-7_8)

Hestrin S, 2011. Neuroscience. The strength of electrical synapses. *Science*, 21;334(6054):315-6. doi: 10.1126/science.1213894

Hofer SB, Mrsic-Flogel TD, Bonhoeffer T, Hübener M. (2009). Experience leaves a lasting structural trace in cortical circuits. *Nature* 457, 313–317. doi:10.1038/nature07487

Kirov SA, Goddard CA, Harris KM. Age-dependence in the homeostatic upregulation of hippocampal dendritic spine number during blocked synaptic transmission. *Neuropharmacology*. 2004 Oct;47(5):640-8. doi: 10.1016/j.neuropharm.2004.07.039

Kirov SA, Harris KM. Dendrites are more spiny on mature hippocampal neurons when synapses are inactivated. *Nat Neurosci*. 1999 Oct;2(10):878-83. doi: 10.1038/13178.

Maletic-Savatic M, Malinow R, Svoboda K. Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science*. 1999 Mar 19;283(5409):1923-7. doi: 10.1126/science.283.5409.1923

O'Neill K, Musgrave IF, Humpage A. Extended Low-Dose Exposure to Saxitoxin Inhibits Neurite Outgrowth in Model Neuronal Cells. *Basic Clin Pharmacol Toxicol*. 2017 Apr;120(4):390-397. doi: 10.1111/bcpt.12701

Pan Y and Monje M, 2020. Activity Shapes Neural Circuit Form and Function: A Historical Perspective. *Journal of Neuroscience*, 40 (5) 944-954. <https://doi.org/10.1523/JNEUROSCI.0740-19.2019>

Stoop R, Poo MM. Synaptic modulation by neurotrophic factors: differential and synergistic effects of brain-derived neurotrophic factor and ciliary neurotrophic factor. *J Neurosci*. 1996 May 15;16(10):3256-64. doi: 10.1523/JNEUROSCI.16-10-03256.1996

Striedter GF. Evolution of the hippocampus in reptiles and birds. *J Comp Neurol*. 2016 Feb 15;524(3):496-517

Vees AM, Micheva KD, Beaulieu C, Descarries L. Increased number and size of dendritic spines in ipsilateral barrel field cortex following unilateral whisker trimming in postnatal rat. *J Comp Neurol*. 1998 Oct 12;400(1):110-24

Glossary [and/or] Abbreviations

AOP Adverse outcome pathway

EPSC Excitatory synaptic currents

KE	Key Event
KERs	Key event relationships
TTX	Tetrodotoxin

KER5: KER749: Hippocampal anatomy altered leads to hippocampal physiology altered

KER749 was extracted from the AOP Wiki on October 30 2023 changes proposed by the WG were agreed with the authors in an EFSA WG meeting.

Key Event Relationship Title

Upstream event: Hippocampal anatomy, Altered

Downstream event: Hippocampal Physiology, Altered

Key Event Relationship Overview

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding	Point of Contact	Author Status	OECD Status
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low	Kevin Crofton (send email)	Open for citation & comment	WPHA/WNT Endorsed

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding	Point of Contact	Author Status	OECD Status
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low	Mary Gilbert (send email)	Under Development: Contributions and Comments Welcome	
Thyroid Receptor Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low	Kevin Crofton (send email)	Under development: Not open for comment. Do not cite	Under Developme
Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent			Katie Paul Friedman (send email)	Open for adoption	Under Developme

Taxonomic Applicability

Term	Scientific Term	Evidence
rat	<i>Rattus norvegicus</i>	Moderate
mouse	<i>Mus musculus</i>	Moderate
human	<i>Homo sapiens</i>	Not Specified

Sex Applicability

Sex	Evidence
Male	High
Female	High

Life Stage Applicability

Term	Evidence
During brain development	High

Key Event Relationship Description

The hippocampus is a highly integrated and organized communication and information processing network with millions of interconnections among its constitutive neurons (see Andersen et al, 2006). Neuronal spines are the primary site of action for synaptic interface between neurons. Although difficult to measure due to their small size, large number and variable shapes, changes in the frequency and structure of dendritic spines of hippocampal neurons has dramatic effects on synaptic physiology and plasticity (Harris et al., 1984). Anatomical integrity at a more macro-level is also essential for physiological function. The connectivity of axons emanating from one set of cells that synapse on the dendrites of the receiving cells must be intact for effective communication between neurons to be possible. Synaptogenesis is a critical step for neurons to be integrated into neural networks during development. Changes in the placement of cells within the network due to delays or alterations in neuronal migration, the absence of a full proliferation of dendritic arbors and spines upon which synaptic contacts are made, and the lagging of transmission of electrical impulses (e.g., due to insufficient myelination) will independently and cumulatively impair synaptic function.

It is also well documented that at the molecular level (mainly based on studies in genetically modified mouse models summarized in [Table XXX](#)[Appendix C table](#)) that disruption in the expression of pre- and post-synaptic proteins involved in the presynaptic vesicular docking, fusion or release of neurotransmitters impair stabilization of synapses

during development. This ultimately alters cytoarchitecture at the macro and ultrastructural level and leads to modification in the physiological integrity of neural circuits in the mature brain. The functional impact of these macro- and micro- alterations hippocampus at the structural level are most often revealed by deficits in neurotransmission and activity-dependent plasticity.

Evidence Collection Strategy

The majority of **Most of** the text in this KER was reviewed and revised as part of the OECD AOP project and approved in 2019. This text was developed using standard literature search procedures set to identify any links between anatomical changes in the hippocampus and subsequent changes in hippocampal physiology. The most important resulting papers were identified as having measured both KE5 and KE6. This was updated by a recent systematic search conducted by EFSA in 2021 and 2022 (see Appendix B of EFSA technical report, 2024). Additional papers, including those using gene models, were identified, and added to the text.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of alterations in hippocampal structure impacting synaptic function and plasticity in the brain is **strong**. Because synaptic transmission in the hippocampus relies on the integrity of contacts and the reliability of electrical and chemical transmission between pre- and post-synaptic neurons, it is well accepted that interference on the anatomical levels will largely impact the functional output on the neurophysiological level (Knowles, 1992; Schultz and Engelhardt, 2014).

Extensive research has provided substantial data on the characteristics supporting a direct link between alterations in neuronal anatomy (axon and dendritic spines morphology, shape and density, vesicular proteins and release, synaptogenesis and neuronal network formation) and neurotransmission, particularly in the context of activity-dependent changes in synaptic strength (synaptic plasticity), best exemplified in the phenomenon of long-term potentiation (LTP). For instance, spine structure is closely linked to synapse function, as the size of spine heads scales with synaptic strength (Matsuzaki et al., 2001; Noguchi et al., 2011). Moreover, the shape and number of spines can be modified by the induction of synaptic plasticity (Matsuzaki et al., 2001; Tønnesen et al., 2014; Zhou et al., 2004). These anatomical alterations in hippocampus lead to changes in the electrophysiological properties of this brain region. Specifically, they serve as physiological readouts of hippocampal function at the synaptic level. The most common physiological readouts were revealed as impairments in basal neurotransmission, synaptic inhibition, and synaptic plasticity (LTP and LTD) (Schnell et al., 2002; Ehrlich & Malinow, 2004; Schmeisser et al., 2012). As detailed in KER4, these same activity-dependent

processes are invoked as mechanistic underpinnings for how neuronal activity impacts affects structure, especially particularly in the developing brain.

Empirical Evidence

Empirical support for this KER is rated as **moderate**. There is no doubt that alterations of the structure of the hippocampus can lead to alterations of its function. Both *in vivo* and *in vitro* studies have demonstrated changes in glial and neuronal cell number or morphology impact physiological function in the hippocampus. Alterations in neurite number, length and complexity have been documented in hippocampal slice cultures with corresponding changes in synaptic function (Hosokawa et al., 1995). Chemical stressors (e.g., prenatal alcohol, developmental Pb exposure, hypoxia), nutritional deficits, and selective lesion models demonstrate a correlative link between altered structure and impaired synaptic function within the hippocampus (Gil-Mohapel et al., 2010; Berman and Hannigan, 2000; Palop et al., 2010; Ieraci and Herrera, 2007). Numerous examples of a direct linkage between hippocampal anatomy and hippocampal physiology are evident in knock out or transgenic mouse models (e.g., Lessman et al., 2011), a few of which are detailed below.

Mutations of the tyrosine kinase gene *Fyn* during development increased the number of neurons in the dentate gyrus and CA subfields of the hippocampus. *Fyn* mutant mice also exhibited impairments in long-term potentiation in hippocampal CA1 whereas two other forms of short-term plasticity remained intact (Grant et al., 1992).

NeurogluinNeoregulin-2 (NRG2) is a growth factor that is highly expressed in the hippocampal dentate gyrus where it contributes to synaptogenesis of newborn granule cells. In hippocampal slice cultures, inducible microRNA targeting strategies have shown that suppression of NRG2 reduced synaptogenesis of inhibitory neurons and impaired dendritic outgrowth and maturation of glutamatergic synapses. These anatomical alterations were accompanied by reductions in the amplitude of excitatory synaptic currents. The magnitude of the impairment was dependent on the timing of the infection and could be eliminated with overexpression of NRG2 in this *in vitro* model (Lee et al., 2015).

Brain-derived neurotrophic factor (BDNF) activation of CREB-activated gene expression plays a documented role in hippocampal synaptogenesis, dendrite formation, and synaptic plasticity in the developing and adult nervous systems (Lessmann et al., 2011; Panja and Bramham, 2014). Jacob is a protein that translocates to the nucleus upon activation of BDNF-dependent pathways and is involved in both neuronal plasticity and neurodegeneration. Hippocampal neurons in culture derived from Jacob/Nsmf knockout mice exhibit shorter neurites with reduced branching and fewer synaptic contacts. This effect was specific to hippocampal neurons, as cortical cells derived from the same animals did not display these abnormalities. *In vivo*, these animals exhibited a reduction of dendritic complexity of CA1 neurons, lower number of branches, and decreased spine

density. Deficits in synaptic plasticity in the form of LTP accompanied these structural impairments (Spilker et al., 2016).

Knockout of PSD-95 (a post-synaptic protein which regulates AMPA-R trafficking and synaptic maturation) impaired long-term depression in CA1 neurons and decreased synaptic strength. Loss of PSD-95 thwarted the developmental increase in the number of functional AMPA-Rs expressing synapsis and prevented developmental changes in spine density and morphology (decreased spine size, a larger number of transient spines that were less stable), arresting synapses in a more immature state (Ehrlich et al., 2007). However, overexpression of PSD-95 increased synaptic strength (by enhancing LTD (Schnell et al., 2002; Ehrlich & Malinow, 2004).

IKK/NF- κ B signaling is critically involved in synapse formation and spine maturation in the adult brain. IKK/NF- κ B blockade in hippocampus of mutant animals was associated with reduced levels of mature spines and postsynaptic proteins (PSD95, SAP97, GluA1), and AMPAR-mediated basal synaptic transmission was suppressed. Exogenous Igf2 (IKK/NF- κ B target) was able to restore synapse density and promote spine maturation (Schmeisser et al., 2012).

In Alzheimer's Disease, amyloid- β protein accumulates in the hippocampus and leads to the formation of amyloid plaques, neuritic dystrophy and aberrant sprouting of axon terminals of the hippocampus. In a developmental germ-line knockout mouse model, high levels of amyloid- β induced aberrant neuronal network excitability and altered innervation of inhibitory interneurons. Deficits in hippocampal plasticity were seen in the dentate gyrus without change in basal levels of synaptic transmission. In contrast, in area CA1, synaptic transmission was impaired while measures of synaptic plasticity remained intact (Palop et al., 2007).

Other evidence for a direct linkage between hippocampal anatomy and hippocampal physiology comes from the area of adult neurogenesis. The neurogenesis process refers to the acquisition of new neurons on the hippocampus of the adult brain and is associated with enhanced hippocampal synaptic function and learning ability (Deng et al., 2010). Manipulations such as caloric restriction, exercise and hormones can enhance neurogenesis and increase synaptic transmission and plasticity (Kapoor et al., 2015; Trivino-Paredes et al., 2016; Deng et al., 2010). A reciprocal relationship also exists whereby increases in hippocampal neural activity serves to increase neurogenesis (Bruel-Jungerman et al., 2007, Bruel-Jungerman et al., 2009, Kameda et al., 2012). Manipulations that decrease hippocampal neurogenesis including exposure to antidepressants, hormone disruption, radiation, genetic ablation, stress, and alcohol are also associated with impaired synaptic function (Herrera et al., 2003; Saxe et al., 2006; Gilbert et al., 2016; Montero-Pedrazuela et al., 2006; Gil-Mohapel et al., 2010).

Temporal Evidence

The temporal nature of this KER is developmental (Seed et al., 2005). This has been demonstrated in multiple studies. A few examples detailed above defined critical periods for the manipulation that alters the structural development of the hippocampus that persists to adulthood to disrupt the synaptic physiology measured in the hippocampus in adulthood (Lee et al., 2015; Grant et al., 1992). A more limited number of 'rescue' experiments have been reported. As described above in Empirical Evidence supporting this KER, Lee et al (2015), using an *in vitro* model, demonstrated impaired synaptogenesis that was dependent on the timing of the infection and could be eliminated with overexpression of NRG2. In Spilker et al. (2016), BDNF application rescued the morphological deficits in hippocampal pyramidal neurons from Jacob/Nsmf mice. Knockout of PSD-95 which altered spine density and morphology resulting in impaired long-term depression in CA1 neurons (decreased synaptic strength) that was rescued by overexpression of PSD-95 (Schnell et al., 2002; Ehrlich & Malinow, 2004). Similarly, in Schmeisser et al (2019) IKK/NF- B blockade in hippocampus of mutant animals reduced levels of mature spines and postsynaptic proteins (PSD95, SAP97, GluA1) and AMPAR-mediated basal synaptic transmission which was restored by exogenous application of Igf2 (IKK/NF- B target).

Dose-Response Evidence

Dose-response data is lacking for this KER. For future research, it is critical to generate data in which the upstream KE is modulated in a 'dose-response' manner to better support the causative relationship.

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are uncertainties. Although several examples are evident to demonstrate direct linkages between alterations in hippocampal anatomy and disruptions in hippocampal physiology, there is not a common cellular mechanism, anatomical insult, or signature pattern of synaptic impairment that defines a common anatomically driven physiological phenotype. In addition, it is also known that there is an interaction between physiological and anatomical development, where anatomy develops first, and can be 'reshaped' by the ongoing maturation of physiological function (e.g., Kutsarova et al., 2017). The scientific understanding of the causative, interactive, and quantitative relationship between the two KEs is currently incomplete.

Known modulating factors

There are currently no known modulating factors.

Quantitative Understanding of the Linkage

The scientific understanding of the causative and quantitative relationship between the two KEs is currently incomplete.

Response-response Relationship

~~Although several examples are evident to demonstrate direct linkages between alterations in hippocampal anatomy and disruptions in hippocampal physiology, there is not one mechanism, one anatomical insult, or one signature pattern of synaptic impairment that accompanies each of these treatments. Information does not exist to develop quantitative relationships between the KEs in this KER. Papers-Publications that utilize knock-out and mutant models have not provided 'dose-response' information for anatomy-physiology relationships.~~

Time-scale

Known Feedforward/Feedback loops influencing this KER

There are currently no known Feedforward/Feedback loops influencing this KER .

Domain of Applicability

~~The majority of~~Most data in support of this KER is from rodent models, supported by limited evidence in humans. The evolutionary conservation of hippocampal anatomy in mammals, birds, and reptiles (see Hevner, 2016; Streidter, 2015) suggests, with some uncertainty, that this KER is also applicable to multiple species.

References

Andersen, P., Morris, R., Amaral, D., Bliss, T., O'Keefe, J. (Editors). The Hippocampus Book. Oxford University Press, 2006. ISBN: 9780195100273

Berman RF, Hannigan JH. Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. Hippocampus. 2000;10(1):94-110.

Bruel-Jungerman E, Davis S, Laroche S (2007) Brain plasticity mechanisms and memory: a party of four. Neuroscientist 13:492-505.

Bruel-Jungerman E, Veyrac A, Dufour F, Horwood J, Laroche S, Davis S (2009) Inhibition of PI3K-Akt signaling blocks exercise-mediated enhancement of adult neurogenesis and

synaptic plasticity in the dentate gyrus. *PLoS ONE* 4(11): e7901. doi:10.1371/journal.pone.000790

~~Deng, W., Aimone, J. & Gage, F. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?. *Nat Rev Neurosci* 11, 339–350 (2010). <https://doi.org/10.1038/nrn2822>~~

Deng, W., Aimone, J. & Gage, F. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?. *Nat Rev Neurosci* 11, 339–350 (2010). <https://doi.org/10.1038/nrn2822>

Ehrlich I, Malinow R (2004), Postsynaptic Density 95 controls AMPA Receptor Incorporation during Long-Term Potentiation and Experience-Driven Synaptic Plasticity. *J Neurosci* 24:916–927

Ehrlich I, Klein M, Rumpel S, Malinow R. 2007, PSD-95 is required for activity-driven synapse stabilization *PNAS*, 104, 4176–4181

Gilbert ME, Goodman JH, Gomez J, Johnstone AF, Ramos RL. Adult hippocampal neurogenesis is impaired by transient and moderate developmental thyroid hormone disruption. *Neurotoxicology*. 2016 Dec 31;59:9-21.

Gil-Mohapel J, Boehme F, Kainer L, Christie BR. Hippocampal cell loss and neurogenesis after fetal alcohol exposure: insights from different rodent models. *Brain Res Rev*. 2010 Sep 24;64(2):283-303.

Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER. Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science*. 1992 Dec 18;258(5090):1903-10.

Harris KM, Teyler TJ. Developmental onset of long-term potentiation in area CA1 of the rat hippocampus. *J Physiol*. 1984 Jan;346:27-48.

Herrera DG, Yague AG, Johnsen-Soriano S, Bosch-Morell F, Collado-Morente L, Muriach M, Romero FJ, Garcia-Verdugo JM (2003) Selective impairment of hippocampal neurogenesis by chronic alcoholism: protective effects of an antioxidant. *Proc Natl Acad Sci U S A* 100:7919-7924.

Hevner RF. Evolution of the mammalian dentate gyrus. *J Comp Neurol*. 2016 524(3):578-94.

Hosokawa T., Rusakov DA, Bliss TVP, and Fine A: Repeated Confocal Imaging of Individual Dendritic Spines in the Living Hippocampal Slice: Evidence for Changes in

Length and Orientation Associated with Chemically Induced LTP. *The Journal of Neuroscience*, August 1995, 75(8): 5580-5573

Ieraci A, Herrera DG. Single alcohol exposure in early life damages hippocampal stem/progenitor cells and reduces adult neurogenesis. *Neurobiol Dis*. 2007 Jun;26(3):597-605.

Kameda M, Taylor CJ, Walker TL, Black DM, Abraham WC, Bartlett PF (2012) Activation of latent precursors in the hippocampus is dependent on long-term potentiation. *Transl Psychiatry* 2:e72.

Kapoor R, Fanibunda SE, Desouza LA, Guha SK, Vaidya VA (2015) Perspectives on thyroid hormone action in adult neurogenesis. *J Neurochem* 133:599-616.

Knowles WD, Normal anatomy and neurophysiology of the hippocampal formation. *J Clin Neurophysiol*. 1992 Apr;9(2):252-63.

Kutsarova E, Munz M, Ruthazer ES. Rules for Shaping Neural Connections in the Developing Brain. *Front Neural Circuits*. 2017. 10:111. doi: 10.3389/fncir.2016.00111.

Lee KH, Lee H, Yang CH, Ko JS, Park CH, Woo RS, Kim JY, Sun W, Kim JH, Ho WK, Lee SH. Bidirectional Signaling of Neuregulin-2 Mediates Formation of GABAergic Synapses and Maturation of Glutamatergic Synapses in Newborn Granule Cells of Postnatal Hippocampus. *J Neurosci*. 2015 Dec 16;35(50):16479-93.

Lessmann V, Stroh-Kaffei S, Steinbrecher V, Edelmann E, Brigadski T, Kilb W, Luhmann HJ. The expression mechanism of the residual LTP in the CA1 region of BDNF k.o. mice is insensitive to NO synthase inhibition. *Brain Res*. 2011. 1391:14-23.

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H. Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. *Nat Neurosci*. 2001 Nov;4(11):1086-92.

Montero-Pedrazuela A, Venero C, Lavado-Autric R, Fernandez-Lamo I, Garcia-Verdugo JM, Bernal J, Guadano-Ferraz A (2006) Modulation of adult hippocampal neurogenesis by thyroid hormones: implications in depressive-like behavior. *Mol Psychiatry* 11:361-371.

Noguchi J, Nagaoka A, Watanabe S, Ellis-Davies GC, Kitamura K, Kano M, Matsuzaki M, Kasai H. In vivo two-photon uncaging of glutamate revealing the structure-function relationships of dendritic spines in the neocortex of adult mice. *J Physiol*. 2011 May 15;589(Pt 10):2447-57.

Palop JJ, Chin J, Roberson ED, Wang J, Thwin MT, Bien-Ly N, Yoo J, Ho KO, Yu GQ, Kreitzer A, Finkbeiner S, Noebels JL, Mucke L. Aberrant excitatory neuronal activity and

compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. *Neuron*. 2007 Sep 6;55(5):697-711.

Panja, D. and C. R. Bramham (2014). "BDNF mechanisms in late LTP formation: A synthesis and breakdown." *Neuropharmacology* 76 Pt C: 664-676. Schultz C, Engelhardt M. Anatomy of the hippocampal formation. *Front Neurol Neurosci*. 2014. 34:6-17

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A*. 2006 Nov 14;103(46):17501-6.

Schmeisser MJ, Baumann B, Johannsen S, Vindedal GF, Jensen V, Hvalby OC, Sprengel R, Seither Maqbool A, Magnutzki A, Lattke M, Oswald F, Boecker TM, Wirth T. 2012, Cellular/Molecular IB Kinase/Nuclear Factor B-Dependent Insulin-Like Growth Factor 2 (Igf2) Expression Regulates Synapse Formation and Spine Maturation via Igf2 Receptor Signaling. *The Journal of Neuroscience*: 32(16):5688 –5703

Schnell E, Sizemore M, Karimzadegan S, Chen L, Bredt DS, Nicoll RA (2002), Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number *Proc Natl Acad Sci USA* 99:13902–13907.

Schultz C, Engelhardt M. Anatomy of the hippocampal formation. *Front Neurol Neurosci*. 2014. 4:6-17.

Seed J, Carney EW, Corley RA, Crofton KM, DeSesso JM, Foster PM, Kavlock R, Kimmel G, Klaunig J, Meek ME, Preston RJ, Slikker W Jr, Tabacova S, Williams GM, Wiltse J, Zoeller RT, Fenner-Crisp P and Patton DE, 2005. Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. *Critical reviews in toxicology* , 35:664-72.

Spilker C, Nullmeier S, Grochowska KM, Schumacher A, Butnaru I, Macharadze T, Gomes GM, Yuanxiang P, Bayraktar G, Rodenstein C, Geiseler C, Kolodziej A, Lopez-Rojas J, Montag D, Angenstein F, Bär J, D' Hanis W, Roskoden T, Mikhaylova M, Budinger E, Ohl FW, Stork O, Zenclussen AC, Karpova A, Schwegler H and Kreutz MR, 2016. A Jacob/Nsmf Gene Knockout Results in Hippocampal Dysplasia and Impaired BDNFSignaling in Dendritogenesis. *PLoS Genetics*. 12(3): e1005907. <https://doi.org/10.1371/journal.pgen.1005907>

Striedter GF. Evolution of the hippocampus in reptiles and birds. *J Comp Neurol*. 2016 Feb 15;524(3):496-517

Tønnesen J, Katona G, Rózsa B, Nägerl UV. Spine neck plasticity regulates compartmentalization of synapses. *Nat Neurosci*. 2014 May;17(5):678-85.

Triviño-Paredes J, Patten AR, Gil-Mohapel J, Christie BR. The effects of hormones and physical exercise on hippocampal structural plasticity. *Front Neuroendocrinol*. 2016; 41:23-43.

Zhou Q, Koichi J. Homma KJ, Poo M., 2004, Shrinkage of Dendritic Spines Associated with Long-Term Depression of Hippocampal Synapses. *Neuron*, 44 (5):749-757

Glossary [and/or] Abbreviations

AhR	Aryl-hydrocarbon receptor
AOP	Adverse outcome pathway
AOP_Wiki	Collaborative Adverse Outcome Pathway Wiki
BDNF	Brain-derived neurotrophic factor
EFSA	European Food Safety Agency
EPSP	Excitatory postsynaptic potential
KEs	Key Events
KERs	Key event relationships
LTD	Long term depression
LTP	Long term potentiation
NIS	Sodium Iodide Symporter
NMDA	n-Methyl-d-aspartate
NRG2	Neuroregulin-2
OECD	Organization for Economic Co-operation and Development
TH	Thyroid Hormone
TR	Thyroid Receptor
WG	Working Group

KER6: KER748: Hippocampal Physiology Altered leads to Cognitive Function Decreased

KER748 was extracted from the AOP Wiki on October 30 2023 changes proposed by the WG were agreed with the authors in an EFSA WG meeting.

Key Event Relationship Title:

Hippocampal Physiology, Altered leads to Cognitive Function, Decreased

Upstream event:

Hippocampal Physiology, Altered

Downstream event:

Cognitive Function, Decreased

Key Event Relationship Overview

AOPs Referencing Relationship

P Name	Adjacency	Weight of Evidence	Quantitative Understanding	Point of Contact	Author Status	OECD Status
Inhibition of Thyroperoxidase and Subsequent Adverse	adjacent	High	Moderate	Kevin Crofton (send email)	Open for citation & comment	WPHA/WNT Endorsed

Neurodevelopmental Outcomes in Mammals						
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Low	Mary Gilbert (send email)	Under Development: Contributions and Comments Welcome	
Thyroid Receptor (TR) Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Moderate	Kevin Crofton (send email)	Under development: Not open for comment. Do not cite	Under Development
AhR activation in the liver leading to Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Low	Low	Prakash Patel (send email)	Under development: Not open for comment. Do not cite	
AhR activation in the thyroid leading to Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	Moderate	Moderate	Prakash Patel (send email)	Under development: Not open for comment. Do not cite	

Taxonomic Applicability

Term	Scientific Term	Evidence
rat	<i>Rattus norvegicus</i>	High
mouse	<i>Mus musculus</i>	High
humans	<i>Homo sapiens</i>	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Life Stage Applicability

Term	Evidence
During brain development	High

Key Event Relationship Description

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 (Burgess, 2002; Martin et al., 2000; Sweatt, 2016). ~~A large number of~~ Many studies with a variety of techniques and approaches (e.g. nutritional and chemical stressors, gene knockouts) have linked hippocampal functional deficits to decreased spatial ability, context learning, and fear learning. Study of human disease states and conditions where hippocampal function is impaired (i.e., brain trauma, Alzheimer's disease, temporal lobe epilepsy, Down's Syndrome), and imaging studies of hippocampal activation during memory challenge, makes irrefutable that the hippocampus is essential for specific types of cognitive abilities. Decades of animal research has reinforced this assertion.

There are many forms of synaptic plasticity and numerous ways in which physiological function of neural circuits can be assessed. Similarly, there are many forms of learning and memory relying on different brain regions and and multiple tasks and specifics associated with these tasks that vary from laboratory to laboratory. An emerging field of computational cognitive neuroscience lies at the intersection of computational neuroscience, machine learning and neural network theory. These computational and theoretical frameworks support the participation of hippocampal synaptic transmission and plasticity in learning and memory in animals and humans (for review see: Ashby and Helie, 2012).

Evidence Collection Strategy

The majority of the text in this KER was reviewed and revised as part of the OECD AOP project and approved in 2019. This text was developed using standard literature search procedures set to identify any links between anatomical changes in the hippocampus and subsequent changes in hippocampal physiology. The most important resulting papers were identified as having measured both KE5 and KE6. This was updated by a recent systematic search conducted by EFSA in 2021 and 2022 (see Appendix B of EFSA technical report, 2024). Additional papers, including those using gene models, were identified, and added to the text.

Evidence supporting this KER

The weight of evidence for physiological hippocampal function and episodic memory in humans and the animal analogue, spatial and fear-based context learning, is strong. Seminal studies over the past 60 years firmly established the cellular basis of behavior with synaptic plasticity (long term potentiation and long-term depression, LTP and LTD respectively). Recent work has provided details on the local hippocampal circuitry needed for memory formation and behavioral change (Sweatt, 2016). In humans, virtual reality experiments in large-scale spatial contexts show the convergence of spatial memory performance in normal patients with fMRI of the hippocampus clearly demonstrating the essentiality of hippocampal function to spatial learning (Burgess, 2002). This assertion is consistent with a wealth of animal data on hippocampal learning and memory. In rodent models, functional impairment of the hippocampus assessed using electrophysiological techniques is correlated with deficits in spatial memory typically assessed using mazes, and memory for context often assessed in fear-based learning paradigms (O'Keefe and Nadel, 1978; Clark et al., 2000; Squire, 2004; Eichenbaum, 2000; Panja and Bramham, 2014).

Biological Plausibility

The biological plausibility of the KER is rated as **strong**. It is well accepted that the normal hippocampal function is critical for the acquisition and memory of context and spatially mediated tasks in rodents and humans (Sweatt, 2016).

Empirical Evidence

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 (Gruart and Delgado-Garcia, 2007). Impairments in hippocampal function induced by drugs, chemicals, lesions, nutritional deficiencies, mutant or knock out models that cause changes in synaptic transmission, plasticity, and hippocampal network activity, are coincident with deficits in spatial and context-based fear learning (O'Keefe and Nadel, 1978; Bannerman et al., 2014; Lynch, 2004; Verret et al., 2012). Similarly, treatments found to enhance or facilitate hippocampal synaptic transmission and plasticity are associated with improved learning and memory (Deng et al., 2010; Novkovic et al., 2015; Andrade et al., 2015; Trivino-Paredes et al., 2016). A few examples of a large literature are briefly summarized below.

It is well known that n-Methyl-d-aspartate (NMDA)-mediated glutamatergic synaptic transmission is essential for the induction of hippocampal synaptic plasticity in the form of LTP. Blockade of this form of plasticity by selective NMDA-receptors blockers impairs LTP and hippocampal tests of learning and memory (reviewed in Sweatt, 2016). Perturbation of hippocampal plasticity and impaired spatial learning have been reported in adult offspring following prenatal ethanol exposure (An and Zhang, 2015). Developmental morphine exposure caused decreasea decrease in the amplitude and slope of fEPSC sand inhibition of LTP in CA1 neurons fEPSPs that resulted in decreased maze performance (Aghighi et al., 2019). Developmental nutrition deficiency and hypoxic stress are both associated with changes in synaptic structure, altered EPSPs, and hippocampal based cognitive behaviors (Dumets et al., 2020; Zhuravin et al., 2019). Rodent models of developmental TH insufficiency are associated with, impairments in hippocampal synaptic transmission and plasticity and are coincident with deficits in learning tasks that require the hippocampus (Opazo et al., 2008; Gilbert and Sui, 2006, Gilbert, 2011, Gilbert et al., 2016).

There are also a-number-ofseveral mutant mouse models that have linked changes in hippocampal physiology with alteration in cognitive behaviors. The fyn mutant mouse (fyn is a tyrosine kinase pathway) displays impairments in hippocampal synaptic transmission and plasticity, as well as spatial learning deficits (Grant et al., 1992). Brain-derived neurotrophic factor (BDNF) knockout animals exhibit synaptic plasticity deficits and learning impairments (Aarse et al., 2016; Panja and Bramham, 2014). In the Jacob/Nfsm model which also exhibits pronounced alterations in BDNF-mediated signaling, hippocampal synaptic transmission and plasticity impairments were accompanied by deficits in contextual fear conditioning and novel location recognition tasks (Spilker et al., 2016). The aryl-hydrocarbon receptor (AhR) knockout was shown to decrease hippocampal mossy fibers and also impair maze performance (Powers et al., 2005).

Knockout of SALM4/Lrfn3, a synaptic adhesion molecule that modulates NMDA receptor function, increases NMDA-mediated currents and enhances contextual fear memory. In this model, control level of performance could be restored *via* treatment with fluoxetine, a-selective serotonin reuptake inhibitor (Lie et al., 2021). Finally, a knockout of LIMK-1, a

kinase associated with actin dynamics⁷ was shown to alter hippocampal spine morphology and LTP, with subsequent changes in fear behaviors and a spatial learning task (Meng et al., 2002).

In humans, hippocampal physiology assessed using neuroimaging reveals activation of hippocampus upon engagement in spatial learning and episodic memory providing a direct linkage of these two specific KEs (Burgess, 2002). In fMRI studies of congenitally hypothyroid children, or children born to women with altered thyroid function during pregnancy, changes in hippocampal activity patterns during memory encoding and retention were observed and associated with memory impairments (Wheeler et al., 2012; 2015; Willoughby et al., 2013; 2014).

Temporal Evidence

The temporal nature of this KER is developmental (Seed et al., 2005). This has been demonstrated in multiple studies. It is well-recognized that there are critical developmental windows for disruption of the functional development of the hippocampus and the integrity of this structure is essential for later development of spatial ability, context learning, and fear learning. A wealth of studies ~~have~~has shown correlation between hippocampal LTP and spatial learning performance, as well as the role of glutamatergic synaptic transmission and BDNF-mediated signaling pathways in these processes (Bramham, 2007; Andero et al., 2014; Morris et al., 1986; Sweatt, 2016; Migaud et al., 1998). Although studies on reversibility are relatively ~~rare, but~~rare, but rare, a few examples of deficits in hippocampal synaptic transmission and plasticity documented in knockout mouse models are described above. In addition, in slices from BDNF knockout animals, physiological function can be rescued with recombinant BDNF (Patterson et al., 1996).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. It is a ~~widely held~~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 of learning and memory, and the complexity of synaptic interactions in even the simplest brain circuit.

Known modulating factors

There are currently no known modulating factors.

Quantitative Understanding of the Linkage

Information does not exist to develop quantitative relationships between the KEs in this KER.

Response- response Relationship

Limited dose-response information is available. Mutation and knockout mouse models are not conducive to examination of varying levels of impairment at the physiological or behavioral level. Studies have investigated dose-dependency of impairments in hippocampal electrophysiological and behavior have been reported in animals suffering from developmental TH insufficiency (e.g., Gilbert and Sui, 2006; Gilbert, 2011; Gilbert et al., 2016).

Known Feedforward/Feedback loops influencing this KER

There are currently no known feedforward/Feedback loops influencing this KER

Domain of Applicability

Most work has been conducted with rodent models. Sex-specific differences in sensitivity to disruption and sex-dependent differences in behavioral performance of hippocampal tasks have been reported in both rodent models and human studies.

References

Aarse J, Herlitze S, Manahan-Vaughan D. The requirement of BDNF for hippocampal

synaptic plasticity is experience-dependent. *Hippocampus*. 2016 Jun;26(6):739-51.

Aghighi F, Mohammadifar M, Banafsheh HR, Salami M, Talaei SA. Behavioral and electrophysiological aspects of cognition in neonate rats lactated by morphine addicted mothers. *Iran J Basic Med Sci* 2019; 22:1059-1064. doi: 10.22038/ijbms.2019.36892.8789

An L, Zhang T. Prenatal ethanol exposure impairs spatial cognition and synaptic plasticity in female rats. *Alcohol*. 2015 Sep;49(6):581-8.

Andero R, Choi DC, Ressler KJ. BDNF-TrkB receptor regulation of distributed adult neural plasticity, memory formation, and psychiatric disorders. *Prog Mol Biol Transl Sci*. 2014. 122:169-92.

Andrade-Talavera Y, Benito I, Casañas JJ, Rodríguez-Moreno A, Montesinos ML. Rapamycin restores BDNF-LTP and the persistence of long-term memory in a model of Down's syndrome. *Neurobiol Dis*. 2015. 82:516-25

Ashby FG, Helie S. The Neurodynamics of Cognition: A Tutorial on Computational Cognitive Neuroscience. *J Math Psychol*. 2011 Aug 1;55(4):273-289.

Bannerman DM, Sprengel R, Sanderson DJ, McHugh SB, Rawlins JNP, Monyer H, Seeburg PH (2014) Hippocampal synaptic plasticity, spatial memory and anxiety. *Nat Rev Neurosci* 15:181-192.

Bramham CR. Control of synaptic consolidation in the dentate gyrus: mechanisms, functions, and therapeutic implications. *Prog Brain Res*. 2007. 163:453-71.

Burgess N (2002) The hippocampus, space, and viewpoints in episodic memory. *Q J Exp Psychol A* 55:1057-1080. Clark RE, Zola SM, Squire LR. Impaired recognition memory in rats after damage to the hippocampus. *J Neurosci*. 2000 Dec 1;20(23):8853-60.

Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory *Nat Rev Neurosci* 11:339-350.

Dumetz F, Ginieis R, Bure C, Marie A, Alfos S, Pallet V, Bosch-Bouju C. Neuronal morphology and synaptic plasticity in the hippocampus of vitamin A deficient rats. *Nutr Neurosci*. 2022 Apr;25(4):779-790. doi: 10.1080/1028415X.2020.1809877. Epub 2020 Sep 12. PMID: 32924835.

Eichenbaum H. (2000). A cortical-hippocampal system for declarative memory. *Nature reviews. Neuroscience*, 1(1), 41–50. <https://doi.org/10.1038/35036213>

Gilbert ME (2011) Impact of low-level thyroid hormone disruption induced by

propylthiouracil on brain development and function. *Toxicol Sci* 124:432-445.

Gilbert ME, Sanchez-Huerta K, Wood C (2016) Mild Thyroid Hormone Insufficiency During Development Compromises Activity- Dependent Neuroplasticity in the Hippocampus of Adult Male Rats. *Endocrinology* 157:774-787.

Gilbert ME, Sui L (2006) Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. *Brain Res* 1069:10-22.

Grant SG, O'Dell TJ, Karl KA, Stein PL, Soriano P, Kandel ER. Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science*. 1992 Dec 18;258(5090):1903-10.

Gruart A, Delgado-García JM. Activity-dependent changes of the hippocampal CA3-CA1 synapse during the acquisition of associative learning in conscious mice. *Genes Brain Behav*. 2007 Jun;6 Suppl 1:24-31.

Lie E, Yeo Y, Lee EJ, Shin W, Kim K, Han KA, Yang E, Choi TY, Bae M, Lee S, Um SM, Choi SY, Kim H, Ko J, Kim E. SALM4 negatively regulates NMDA receptor function and fear memory consolidation. *Commun Biol*. 2021 Sep 29;4(1):1138. doi: 10.1038/s42003-021-02656-3. PMID: 34588597; PMCID: PMC8481232.

Lynch, M.A. (2004). Long-Term Potentiation and Memory. *Physiological Reviews*. 84:87-136.

Martin SJ, Grimwood PD, Morris RG. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci*. 2000. 23:649-711.

Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z. Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron*. 2002 Jul 3;35(1):121-33. doi: 10.1016/s0896-6273(02)00758-4. PMID: 12123613.

Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG. Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature*. 1998 Dec 3;396(6710):433-9.

Morris RG, Frey U. Hippocampal synaptic plasticity: role in spatial learning or the automatic recording of attended experience? *Philos Trans R Soc Lond B Biol Sci*. 1997 Oct 29;352(1360):1489-503. Review

Novkovic T, Mittmann T, Manahan-Vaughan D. BDNF contributes to the facilitation of

hippocampal synaptic plasticity and learning enabled by environmental enrichment. *Hippocampus*. 2015 Jan;25(1):1-15.

O'Keefe, J. and Nadel, L. (1978). The Hippocampus as a Cognitive Map. Oxford: Oxford University Press.

Opazo MC, Gianini A, Pancetti F, Azkconea G, Alarcón L, Lizana R, Noches V, Gonzalez PA, Marassi MP, Mora S, Rosenthal D, Eugenin E, Naranjo D, Bueno SM, Kalergis AM, Riedel CA (2008), Maternal hypothyroxinemia impairs spatial learning and synaptic nature and function in the offspring. *Endocrinology* 149:5097-5106

Panja, D. and C. R. Bramham (2014). "BDNF mechanisms in late LTP formation: A synthesis and breakdown." *Neuropharmacology* 76 Pt C: 664-676.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron*. 1996 Jun;16(6):1137-45.

Powers BE, Lin TM, Vanka A, Peterson RE, Juraska JM, Schantz SL. Tetrachlorodibenzo-p-dioxin exposure alters radial arm maze performance and hippocampal morphology in female AhR mice. *Genes Brain Behav.* 2005 Feb;4(1):51-9. doi: 10.1111/j.1601-183X.2004.00098.x. PMID: 15660668.

Schultz C, Engelhardt M, Anatomy of the hippocampal formation. *Front Neurol Neurosci*. 2014. 34:6-17

Seed J, Carney EW, Corley RA, Crofton KM, DeSesso JM, Foster PM, Kavlock R, Kimmel G, Klaunig J, Meek ME, Preston RJ, Slikker W Jr, Tabacova S, Williams GM, Wiltse J, Zoeller RT, Fenner-Crisp P, Patton DE. Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. *Crit Rev Toxicol*. 2005 35:664-72.

Spilker C, Nullmeier S, Grochowska KM, Schumacher A, Butnaru I, Macharadze T, Gomes GM, Yuanxiang P, Bayraktar G, Rodenstein C, Geiseler C, Kolodziej A, Lopez-Rojas J, Montag D, Angenstein F, Bär J, D'Hanis W, Roskoden T, Mikhaylova M, Budinger E, Ohl FW, Stork O, Zenclussen AC, Karpova A, Schwegler H, Kreutz MR. A Jacob/Nsmf Gene Knockout Results in Hippocampal Dysplasia and Impaired BDNF Signaling in Dendritogenesis. *PLoS Genet*. 2016 Mar 15;12(3):e1005907

Squire LR 2004. Memory systems of the brain: A brief history and current perspective. *Neurobiology of Learning and Memory*, 82: 171-177

Sweatt JD. Neural plasticity and behavior - sixty years of conceptual advances. *J Neurochem*. 2016 Oct;139 Suppl 2:179-199. doi: 10.1111/jnc.13580. Review. PubMed

PMID: 26875778.

Triviño-Paredes J, Patten AR, Gil-Mohapel J, Christie BR. The effects of hormones and physical exercise on hippocampal structural plasticity. *Front Neuroendocrinol*. 2016; 41:23-43.

Verret L, Mann EO, Hang GB, Barth AM, Cobos I, Ho K, Devidze N, Masliah E, Kreitzer AC, Mody I, Mucke L, Palop JJ. Inhibitory interneuron deficit links altered network activity and cognitive dysfunction in Alzheimer model. *Cell*. 2012 Apr 27;149(3):708-21.

Wheeler SM, McAndrews MP, Sheard ED, Rovet J (2012) Visuospatial associative memory and hippocampal functioning in congenital hypothyroidism. *J Int Neuropsychol Soc* 18:49-56.

Wheeler SM, McLelland VC, Sheard E, McAndrews MP, Rovet JF (2015) Hippocampal Functioning and Verbal Associative Memory in Adolescents with Congenital Hypothyroidism. *Front Endocrinol (Lausanne)* 6:163.

Willoughby KA, McAndrews MP, Rovet J (2013) Effects of early thyroid hormone deficiency on children's autobiographical memory performance. *J Int Neuropsychol Soc* 19:419-429.

Willoughby KA, McAndrews MP, Rovet JF (2014) Effects of maternal hypothyroidism on offspring hippocampus and memory. *Thyroid* 24:576-584.

Zhuravin IA, Dubrovskaya NM, Vasilev DS, Postnikova TY, Zaitsev AV. Prenatal hypoxia produces memory deficits associated with impairment of long-term synaptic plasticity in young rats. *Neurobiol Learn Mem*. 2019 Oct;164:107066. doi: 10.1016/j.nlm.2019.107066. Epub 2019 Aug 7. PMID: 31400467.

Glossary [and/or] Abbreviations

AhR	Aryl-hydrocarbon receptor
AOP	Adverse outcome pathway
AOP-Wiki	Collaborative Adverse Outcome Pathway Wiki
BDNF	Brain-derived neurotrophic factor
EFSA	European Food Safety Agency
EPSP	Excitatory postsynaptic potential
KEs	Key Events

KERs	Key event relationships
LTD	Long term depression
LTP	Long term potentiation
NIS	Sodium Iodide Symporter
NMDA	n-Methyl-d-aspartate
OECD	Organization for Economic Co-operation and Development
TH	Thyroid Hormone
TR	Thyroid Receptor

Overall assessment of the AOP

Determination of confidence in the overall AOP as a basis to support specific regulatory application relies on the biological plausibility, empirical support, and quantitative understanding of the KERs, as well as the evidence supporting essentiality of the KEs. Table 1 provides an overall summary of the weight of evidence based on the evaluations of the individual linkages from the Key Event Relationship pages. It indicates how biological plausibility and empirical evidence improved with the new work reported here (e.g. from moderate to strong of biological plausibility of KER749).

Please, refer to Appendix B1. Statistical Analysis report for the description of the methodology and individual assessment in the Expert Knowledge Elicitation.

Table 1. Summary table with the assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements and Expert Knowledge Elicitation. *KER1. Binding to VGSC leads to disruption of sodium channel gate kinetics; KER2. Disruption of sodium channel gate kinetics leads to disruption of action potential; KER3. Disruption of action potential leads to altered neurotransmission during development; KER4. Altered neurotransmission during development leads to hippocampal anatomy altered; KER749. Hippocampal anatomy altered leads to hippocampal physiology altered; KER748. Hippocampal physiology altered leads to cognitive function decrease.*

	KER1	KER2	KER3	KER4	KER749 with new data	KER748 with new data
	Direct KER	Direct KER				

Biological Plausibility	STRONG	STRONG	STRONG	STRONG	STRONG	From moderate to STRONG	From moderate to STRONG
Empirical Evidence	STRONG	STRONG	MODERATE	MODERATE	MODERATE	From moderate to MODERATE-STRONG	From moderate to STRONG

In addition, The following summary tables for:

1. Support for Biological Plausibility of KERS
2. Support for Essentiality of KEs
3. Empirical Support for KERs

Are available in **Appendix E**.

Domain of Applicability

Chemicals: This AOP applies to a wide range of chemicals that binds to VGSC. Well recognized prototypical stressors include natural toxins, TTX (classic stressor), pyrethrins and pyrethroids.

Pyrethrins and Pyrethroids

Natural toxins, produced by animalanimals, plantplants and microorganisms, target VGSCs through diverse strategies developed over millions of years of evolution. The sodium transients can be antagonised by TTX (tetrodotoxin) (Káradóttir et al., 2008; Berrett et al., 2017) which is the classic stressor. Classic and well-studiedwell-studied stressors for VGSCs are pyrethroid insecticides. Indeed, it is well known and accepted that pyrethroids bind to the α α subunit of the neuronal VGSC (Trainer et al., 1997; Smith et al., 1997; Smith and Soderlund, 1998, 2001; Catterall et al., 2007; Cao et al., 2011). Mutations in the α α subunit of both insects (Lee and Soderlund, 2001; Smith et al., 1997) and mammals (Vais et al., 2000, 2001; Wang et al., 2001) alter the sensitivity of VGSCs to pyrethroids, supporting the conclusion that pyrethroid interact with the α α subunit (Shafer et al., 2005). The β subunit has been observed to modulate the affinity of pyrethroid interaction with the channel (Smith and Soderlund, 1998). Further work indicates that deltamethrin effects on sodium currents were dependent on subunit-combinations and the embryonically expressed $\text{Na}_v1.3/\beta 3$ channels were more

sensitive than the $\text{Na}_v1.2/\beta 1$ channels expressed in adulthood. Moreover, the $\text{Na}_v1.3/\beta 3$ channels were particularly sensitive to cyano-containing pyrethroids (type II pyrethroids, e.g., cypermethrin, β -cyfluthrin, esfenvalerate and fenpropathrin) but not for the type I pyrethroids permethrin and tetramethrin (Meacham et al. 2008). Additional studies demonstrated that orthologous channels with a high degree of amino acid sequence conservation differ in both their functional properties and their sensitivity to pyrethroid insecticides. Thus, e.g. human $\text{Na}_v1.3$ channels are not only less sensitive than the rat $\text{Na}_v1.3$ channels but also less sensitive than the relatively less sensitive rat $\text{Na}_v1.2$ channels (Tan and Soderlund, 2009, Bal-Price et.al., 2008).

However, the action of pyrethroid insecticides on sodium channels in invertebrate and vertebrate nerve preparation has been widely documented over the past decades and extensively and critically summarised in numerous reviews (Soderlund et al., 2002; Chahine, 2018). Based on their chemical structure and clinical symptoms of toxicity, pyrethroids are classified in type I and type II. Following the binding to a VGSC specific isoform/s, pyrethroids slow down the activation (or opening), of VGSC. In addition, they reduce the rate of VGSC inactivation (or closing) and shift to the membrane potentials at which VGSC activate (or open) to a more hyperpolarised state (Narahashi, 1996). As a result, sodium channels open at more hyperpolarised potential and remain open for longer, allowing an increased influx of sodium ions that can eventually depolarise the neuronal membrane. Type II pyrethroids prolong VGSCs inactivation more than type I pyrethroids, leading to a depolarisation-dependent block. These differences in channel open times contribute to the distinct toxicological profiles of these chemicals (Verschoyle and Aldridge 1980 Ray, 2001). See Figure 5 below from Shafer et al. (2005), [summarises the effects of pyrethroids on individual channels, whole-cell sodium currents and action potentials.](#)

-

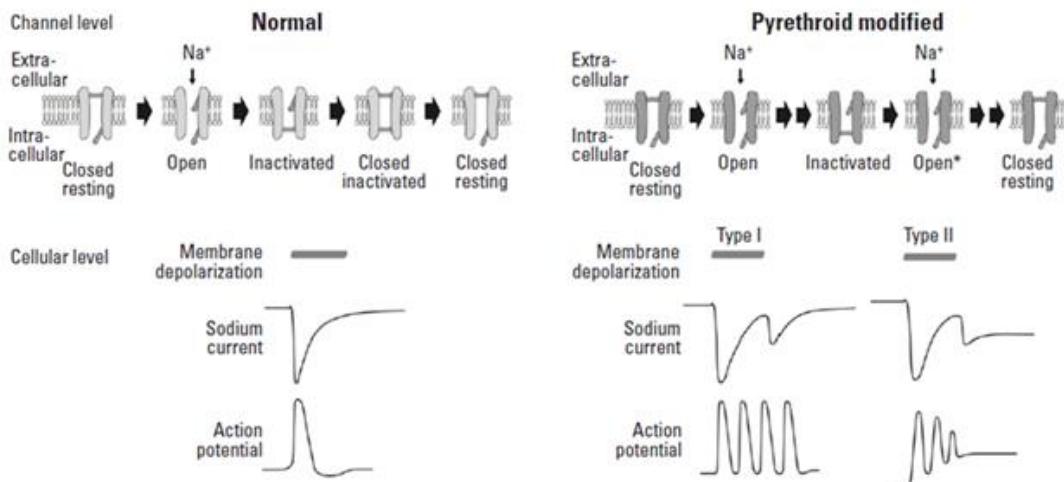


Figure 5: Pyrethroid effects on neuronal excitability. Pyrethroids inhibit the function of 'gates' that control sodium flux through VGSC, delaying inactivation (indicated by the double arrow between states) of the channel and allowing continued sodium flux. After depolarisation ends, pyrethroid-mediated VGSC remain open, resulting in a 'tail' current. Type I pyrethroids action results in a series of

action potentials, while type II pyrethroids cause greater membrane depolarisation, leading to a depolarisation-dependent block. Source: Shafer et al., 2005.

Figure 5 summarises the effects of pyrethroids on individual channels, whole-cell sodium currents and action potentials.

Taxonomic Applicability

Term	Scientific Term	Evidence
rat	<i>Rattus norvegicus</i>	High
mouse	<i>Mus musculus</i>	High
humans	<i>Homo sapiens</i>	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Life Stage Applicability

Life stage: during brain development (embryonic, fetal **infancy**, childhood, adolescence developmental periods)

Essentiality of the Key Events

In accordance with the OECD AOP Handbook the essentiality addresses the impact of manipulation of a given KE on the downstream sequence of KEs defined for the AOP.

It is noted that for this AOP it is widely accepted that each of the key events is essential. In addition, a large **amountnumber** of publications using knock-out methods were retrieved for the different KEs.

Although they do not provide conclusive evidence on essentiality they have been compiled and used for both essentiality and biological plausibility of the KERs (see **Table 3** in Appendix E).

The mutation studies addressing the KERs within this AOP have been carried out both *in vitro* and *in vivo*. Specifically, for KER4 these types of studies have been conducted in knockout mouse models. These studies combine electrophysiological analyses of acute brain slices with methods (e.g., immunohistochemistry) to characterize role of knockout proteins in hippocampal function. Since KE3 and KE4 are usually measured in the same study, it is difficult to determine which one occurs first (KE_{up}) and which occurs later (KE_{down}). As a result, no firm conclusion can be drawn on essentiality, but this evidence is considered proof of it.

AOP Known Modulating Factors

Not applicable/not investigated in detail.

Quantitative Understanding of the KERs

For the current AOP, quantitation of the relationships between KEs is limited. For KER 1 and KER 2, several software packages are available and give a clear and simple description of the voltage and current clamp methods. The software allows setting Na conductance levels and predicts resultant nerve action potentials. These models could be used to estimate the quantitative link between alterations in VGSC kinetics and action potential generation or disruption (MIE to KE2 in this AOP). In addition, as reported in this AOP, the quantitative relationship between the alteration of VGSC kinetics and the action potential generation has been modelled for tetramethrin but not for other pyrethroids. The timescale for the response-response of KER1 and KER2 should be considered as immediate.

Models for quantification of the remaining downstream part of the AOP are not currently available. For KER3, quantification could be feasible if future research uses the methods described in this AOP. By applying these methods, it would be possible to calculate the response-response relationship between the concentration-dependent perturbation of the action potential and the concentration-dependent downstream effect in the NNF assay. This investigation could be done at different stages of development.

A similar experimental approach could be taken to define the quantitative relationship between all remaining downstream KEs and the AO. However, different methodologies and metrics may be needed depending on the type of neuron, brain region and function in the central nervous system.

Considerations for Potential Applications of the AOP

The development of the new KE (KE 2005) referred to us 'Altered neurotransmission during development' has been a critical knowledge compilation in this AOP (see appendix C for detailed information on this KE and its KER). The regulatory relevance of the *in vitro* testable KE "Altered neurotransmission during development" is now further supported by the characterization of mechanistic KERs within this augmented AOP. These KERs link the KEs "altered hippocampal anatomy" and "altered

hippocampal physiology” to the AO “decreased cognitive function”. The biological plausibility and empirical evidence supporting these KERs have been assessed as moderate to strong.

This KE occurs in all life stages. As the balance between excitatory and inhibitory neurotransmission shapes hippocampal circuitry, any perturbation of this balance can lead to abnormal network activity (Cherubini et al., 2021). The methods and test systems used to measure abnormal network activity are similar in developmental or adult life stages. In addition, upstream KEs and pathways occurring in all life stages can lead to alteration in neurotransmission. **However, in this AOP, KE 2005 pertains to the developmental period, because it is recognized that the biological and toxicological consequences can be different when disruption of neurotransmission occurs during development, versus adult life stages.** It is well recognized that an infant’s brain contains more neurons at birth than that of an adult, and the developing brain undergoes remarkable remodelling to achieve mature neural circuits via processes like apoptosis and synaptic pruning. As brain development matures further activity-dependent remodelling will strengthen circuits that prove more relevant and weaken others that are less frequently used. Such remodelling is more prominent in the first 2 years of life in humans and again during adolescence, with neural activity being a key driver for synaptic pruning. Thus, disruption of the formation of precise neural circuits during critical stages of brain development (i.e., perinatal and adolescence) may underlie neurodevelopmental disorders (Faust et al., 2021; see KER4 description and life stage applicability).

In mammals, it is well known that activity-dependent neuronal remodelling and the timing of this process depends on the brain region and cellular subtypes. This AOP focused on the hippocampal region (as detailed in Appendix C) since hippocampal circuits have been more extensively studied, particularly in relation to regulated chemicals. Furthermore, the hippocampus has been causally linked to measurable AOs (e.g., learning and memory) in rodent models. While several model circuits for studying activity-dependent neuronal remodelling are available for many brain regions, future work is required to develop an AOP and KERs for other brain areas.

There are uncertainties in this new chemically agnostic AOP, including but not limited to knowledge gaps regarding quantitative relationships between KEs and the subsequent adverse impacts on cognitive functions, species extrapolation issues common to all animal based AOPs, and possible lower sensitivity of rodent cognition models commonly used in regulatory studies. This also holds true for developing AOPs for other brain regions, since this AOP is focused on the AO of altered hippocampal-based cognitive function. However, regarding the utility of the MEA-based neural network formation (NNF) assay (OECD, 2023) for use in chemical regulation, it is important to note that it uses cortical cell cultures. Thus, an effect on the NNF assay may not necessarily correlate with changes in the hippocampal-based spatial cognitive tests commonly used in regulatory *in vivo* DNT studies. It is biologically highly plausible that disturbed cortical cell based NNF generalizes to an adverse effect within other brain regions. For chemical regulation the derivation of relevant PoDs is more important than the prediction of any specific neurodevelopmental disorder at the organism level.

The newly developed KE 2005 can be measured using many methodologies that examine neural connectivity (i.e., neurotransmission), including the *in vitro* NNF assay. A standardized NNF test system to assess the potential impact of chemical exposure on neural network formation and function has been developed using rodent cortical neurons (Frank et al., 2017). This NNF assay is considered valid,

biologically relevant and reliable by OECD (OECD, 2023) and the US EPA (US EPA, 2020a,b). An analysis for the regulatory use of the rodent primary cortical cell-based NNF assay and the additional 16 *in vitro* DNT assays has also been performed, and this may be contextualized with the uncertainties for *in vivo* data based uncertainties (Paparella et al., 2020).

The NNF assay represents a developing and relatively complex *in vitro* multi-cellular test system that includes many key neurodevelopmental processes, and provides a readout of neurophysiological function measured by changes in synaptic activity (*i.e.*, general network activity, network bursting, network connectivity). If such activity is disturbed, it is likely caused by one or more upstream KEs (in this linear AOP or in a potential AOP network) that have previously been disturbed and not compensated at the (multi)cellular level. If these functional *in vitro* changes are large enough, they will disrupt neurological functions in an organism, ultimately eliciting negative effects. Within experimental systems, a positive response often holds greater regulatory relevance than a negative one, be it *in vitro* or *in vivo*. This is because none of these systems fully encompass all aspects of human higher cerebral functions, or the characteristics exhibited by humans in their natural state, including aspects such as metabolism, kinetics, molecular and cellular characteristics. Thus, positive effects observed in *in vitro* models, or in rodent *in vivo* studies, should be considered indicators of toxicity. Their impact in real life human conditions depends on additional factors such as (epi)genetic background, socioeconomic status, diet, lifestyle, stress, infections and chemical co-exposures.

The direct regulatory relevance of these disruptions, if integrated with other toxicological information, can be used to derive a PoD, which will be the basis for setting a health-based guidance value. To facilitate regulatory use in decision making, an agreed tiered testing strategy approach for use of *in vitro* data would be helpful. This approach should include the MEA-based NFF assay as well as the remaining assays in the DNT IVB together with interpretive guidance on the MEA/NFF outcomes for quantitative human health risk assessment.

References

Bal-Price, A. K., Suñol, C., Weiss, D. G., van Vliet, E., Westerink, R. H. S., & Costa, L. G. (2008). Application of *in vitro* neurotoxicity testing for regulatory purposes: Symposium III summary and research needs. *NeuroToxicology*, 29(3), 520-531. <https://doi.org/10.1016/j.neuro.2008.02.008>

Berret E, Barron T, Xu J, Debner E, Kim EJ and Kim JH, 2017. Oligodendroglial excitability mediated by glutamatergic inputs and Nav1.2 activation. *Nature Communications*, 8(1), 1–15. <https://doi.org/10.1038/s41467-017-00688-0>

Catterall, W. A., Cestèle, S., Yarov-Yarovoy, V., Yu, F. H., Konoki, K., & Scheuer, T. (2007). Voltage-gated ion channels and gating modifier toxins. *Toxicon : official journal of the International Society on Toxicology*, 49(2), 124–141. <https://doi.org/10.1016/j.toxicon.2006.09.022>

Chahine M (ed.), 2018. Voltage-gated Sodium Channels: Structure, Function and Channelopathies.246. Springer.

Cherubini E, Di Cristo G, Avoli M. Dysregulation of GABAergic Signaling in Neurodevelopmental Disorders: Targeting Cation-Chloride Co-transporters to Re-establish a Proper E/I Balance. *Front Cell Neurosci*. 2022 Jan 5;15:813441. doi: 10.3389/fncel.2021.813441

Faust, T.E., Gunner, G. & Schafer, D.P. Mechanisms governing activity-dependent synaptic pruning in the developing mammalian CNS. *Nat Rev Neurosci* 22, 657–673 (2021). <https://doi.org/10.1038/s41583-021-00507-y>

Frank CL, Brown JP, Wallace K, Mundy WR, and Shafer TJ, 2017. From the Cover: Developmental Neurotoxicants Disrupt Activity in Cortical Networks on Microelectrode Arrays: Results of Screening 86 Compounds During Neural Network Formation. *Toxicological Sciences*, 160(1), 121–135. <https://doi.org/10.1093/toxsci/kfx169>

Káradóttir R, Hamilton NB, Bakiri Y and Attwell D, 2008. Spiking and nonspiking classes of oligodendrocyte precursor glia in CNS white matter. *Nature Neuroscience*, 11(4), 450–456. <https://doi.org/10.1038/nn2060>

Meacham CA, Brodfuehrer PD, Watkins JA and Shafer TJ , 2008. Developmentally-regulated sodium channel subunits are differentially sensitive to alpha-cyano containing pyrethroids. *Toxicology and applied pharmacology*, 231(3), 273–281. <https://doi.org/10.1016/j.taap.2008.04.017>

Narahashi T, 1996. Neuronal ion channels as the target sites of insecticides. *Pharmacology and Toxicology*, 79(1), 1–14

OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. 2023. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)

Paparella M, Hougaard Bennekou S, and Bal-Price A, 2020. An analysis of the limitations and uncertainties of in vivo developmental neurotoxicity testing and assessment to identify the potential for alternative approaches. *Reproductive Toxicology*, 96; 327-336. <https://doi.org/10.1016/j.reprotox.2020.08.002>

Shafer TJ, Meyer DA and Crofton KM, 2005. Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environmental Health Perspectives*, 113(2), 123–136. <https://doi.org/10.1289/ehp.7254>

Smith TJ and Soderlund DM, 1998. Action of the pyrethroid insecticide cypermethrin on rat brain IIa sodium channels expressed in xenopus oocytes. Neurotoxicology, 19(6), 823–832.

Smith TJ and Soderlund DM, 2001. Potent actions of the pyrethroid insecticides cismethrin and cypermethrin on rat tetrodotoxin-resistant peripheral nerve (SNS/PN3) sodium channels expressed in Xenopus oocytes. Pesticide Biochemistry and Physiology, 70(1), 52–61.
<https://doi.org/10.1006/pest.2001.2538>

Smith TJ, Lee SH, Ingles PJ, Knipple DC and Soderlund DM, 1997. The L1014F point mutation in the house fly Vssc1 sodium channel confers knockdown resistance to pyrethroids. Insect Biochemistry and Molecular Biology, 27(10), 807–812. https://doi.org/10.1016/S0965-1748(97)00065-9

Smith, E and Jonides, J. (1997). Working Memory: A View from Neuroimaging. Cognitive Psychology, 33:5-42.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, and Weiner ML, 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. Toxicology, 171(1), 3–59.

Tan J and Soderlund DM, 2009. Human and rat Nav1.3 voltage-gated sodium channels differ in inactivation properties and sensitivity to the pyrethroid insecticide tefluthrin. Neurotoxicology, 30(1), 81–9. doi: 10.1016/j.neuro.2008.10.008.

Trainer VL, McPhee JC, Boutelet-Bochan H, Baker C, Scheuer T, Babin D, and Catterall WA, 1997. High affinity binding of pyrethroids to the α subunit of brain sodium channels. Molecular Pharmacology, 51(4), 651–657

US EPA., 2020a., Agency Issue Paper: Use of New Approach Methodologies to Derive Extrapolation Factors and Evaluate Developmental Neurotoxicity for Human Health Risk Assessment. EPA HQ OPP 2020-0263

US EPA., 2020b., Peer Review of the Use of New Approach Methodologies (NAMs) to Derive Extrapolation Factors and Evaluate Developmental Neurotoxicity for Human Health Risk Assessment. FIFRA Scientific Advisory Panel Meeting Minutes and Final Report No. 2020-02

Vais H, Atkinson S, Eldursi N, Devonshire AL, Williamson MS, Usherwood PN. A single amino acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides. FEBS Lett. 2000 Mar 24;470(2):135-8.https://doi.org/10.1016/S0014-5793(00)01305-3

Vais H, Williamson MS, Devonshire AL, and Usherwood PN, 2001. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. Pest Management Science, 57(10), 877–88. https://doi.org/10.1002/ps.392Wang et al., 2001

Verschoyle RD, Aldridge WN. Structure-activity relationships of some pyrethroids in rats. Arch Toxicol. 1980 Oct;45(4):325-9. doi: 10.1007/BF00293813. PMID: 7447703.

Bal-Price, A. K., Suñol, C., Weiss, D. G., van Vliet, E., Westerink, R. H. S., & Costa, L. G. (2008). Application of in vitro neurotoxicity testing for regulatory purposes: Symposium III summary and research needs. *NeuroToxicology*, 29(3), 520–531. <https://doi.org/10.1016/j.neuro.2008.02.008>

Káradóttir R, Hamilton NB, Bakiri Y and Attwell D, 2008. Spiking and nonspiking classes of oligodendrocyte precursor glia in CNS white matter. *Nature Neuroscience*, 11(4), 450–456. <https://doi.org/10.1038/nn2060>

Berret E, Barron T, Xu J, Debner E, Kim EJ and Kim JH, 2017. Oligodendroglial excitability mediated by glutamatergic inputs and Nav1.2 activation. *Nature Communications*, 8(1), 1–15. <https://doi.org/10.1038/s41467-017-00688-0>

Smith TJ and Söderlund DM, 1998. Action of the pyrethroid insecticide cypermethrin on rat brain IIa sodium channels expressed in *Xenopus* oocytes. *Neurotoxicology*, 19(6), 823–832.

Smith TJ and Söderlund DM, 2001. Potent actions of the pyrethroid insecticides cismethrin and cypermethrin on rat tetrodotoxin-resistant peripheral nerve (SNS/PN3) sodium channels expressed in *Xenopus* oocytes. *Pesticide Biochemistry and Physiology*, 70(1), 52–61. <https://doi.org/10.1006/pest.2001.2538>

Smith TJ, Lee SH, Ingles PJ, Knipple DC and Söderlund DM, 1997. The L1014F point mutation in the house fly Vssc1 sodium channel confers knockdown resistance to pyrethroids. *Insect Biochemistry and Molecular Biology*, 27(10), 807–812. [https://doi.org/10.1016/S0965-1748\(97\)00065-9](https://doi.org/10.1016/S0965-1748(97)00065-9)

Catterall, W. A., Cestèle, S., Yarov-Yarovoy, V., Yu, F. H., Konoki, K., & Scheuer, T. (2007). Voltage-gated ion channels and gating modifier toxins. *Toxicon : official journal of the International Society on Toxicology*, 49(2), 124–141. <https://doi.org/10.1016/j.toxicon.2006.09.022>

Cherubini E, Di Cristo G, Avoli M. Dysregulation of GABAergic Signaling in Neurodevelopmental Disorders: Targeting Cation Chloride Co-transporters to Re-establish a Proper E/I Balance. *Front Cell Neurosci*. 2022 Jan 5;15:813441. doi: 10.3389/fncel.2021.813441

Chahine M (ed.), 2018. Voltage-gated Sodium Channels: Structure, Function and Channelopathies. 246. Springer.

Faust, T.E., Gunner, G. & Schafer, D.P. Mechanisms governing activity-dependent synaptic pruning in the developing mammalian CNS. *Nat Rev Neurosci* 22, 657–673 (2021). <https://doi.org/10.1038/s41583-021-00507-y>

Frank CL, Brown JP, Wallace K, Mundy WR, and Shafer TJ, 2017. From the Cover: Developmental Neurotoxicants Disrupt Activity in Cortical Networks on Microelectrode Arrays: Results of Screening 86 Compounds During Neural Network Formation. *Toxicological Sciences*, 160(1), 121–135. <https://doi.org/10.1093/toxsci/kfx169>

~~OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery; Series on Testing and Assessment No. 377. 2023. Available at: [https://one.oecd.org/document/ENV/CBC/MONO\(2023\)13/en/pdf](https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf)~~

~~Meacham CA, Brodfuehrer PD, Watkins JA and Shafer TJ, 2008. Developmentally regulated sodium channel subunits are differentially sensitive to alpha-cyano-containing pyrethroids. *Toxicology and applied pharmacology*, 231(3), 273–281. <https://doi.org/10.1016/j.taap.2008.04.017>~~

~~Narahashi T, 1996. Neuronal ion channels as the target sites of insecticides. *Pharmacology and Toxicology*, 79(1), 1–14~~

~~Paparella M, Hougaard Bennekou S, and Bal Price A, 2020. An analysis of the limitations and uncertainties of in vivo developmental neurotoxicity testing and assessment to identify the potential for alternative approaches. *Reproductive Toxicology*, 96, 327–336. <https://doi.org/10.1016/j.reprotox.2020.08.002>~~

~~Smith, E and Jonides, J. (1997). Working Memory: A View from Neuroimaging. *Cognitive Psychology*, 33:5–42.~~

~~Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, and Weiner ML, 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology*, 171(1), 3–59.~~

~~Shafer TJ, Meyer DA and Crofton KM, 2005. Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. *Environmental Health Perspectives*, 113(2), 123–136. <https://doi.org/10.1289/ehp.7254>~~

~~Tan J and Soderlund DM, 2009. Human and rat Nav1.3 voltage-gated sodium channels differ in inactivation properties and sensitivity to the pyrethroid insecticide tefluthrin. *Neurotoxicology*, 30(1), 81–9. doi: 10.1016/j.neuro.2008.10.008.~~

~~Trainer VL, McPhee JC, Boutelet Bochan H, Baker C, Scheuer T, Babin D, and Catterall WA, 1997. High affinity binding of pyrethroids to the α subunit of brain sodium channels. *Molecular Pharmacology*, 51(4), 651–657~~

~~US EPA., 2020a., Agency Issue Paper: Use of New Approach Methodologies to Derive Extrapolation Factors and Evaluate Developmental Neurotoxicity for Human Health Risk Assessment. EPA HQ OPP 2020-0263~~

~~US EPA., 2020b., Peer Review of the Use of New Approach Methodologies (NAMs) to Derive Extrapolation Factors and Evaluate Developmental Neurotoxicity for Human Health Risk Assessment. FIFRA Scientific Advisory Panel Meeting Minutes and Final Report No. 2020-02~~

Vais H, Atkinson S, Eldursi N, Devonshire AL, Williamson MS, Usherwood PN. A single amino acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides. *FEBS Lett.* 2000 Mar 24;470(2):135-8. [https://doi.org/10.1016/S0014-5793\(00\)01305-3](https://doi.org/10.1016/S0014-5793(00)01305-3)

Vais H, Williamson MS, Devonshire AL, and Usherwood PN, 2001. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. *Pest Management Science*, 57(10), 877-88. <https://doi.org/10.1002/ps.392> Wang et al., 2001

Verschoyle RD, Aldridge WN. Structure-activity relationships of some pyrethroids in rats. *Arch Toxicol.* 1980 Oct;45(4):325-9. doi: 10.1007/BF00293813. PMID: 7447703.

Glossary [and/or] Abbreviations

AO	Adverse outcome
AOP	Adverse outcome pathway
DNT	Developmental neurotoxicity
DNT IVB	Developmental Neurotoxicity In Vitro Testing Battery
KEs	Key Events
KERs	Key event relationships
KNP	Key neurodevelopmental processes
MEA	Microelectrode assay
MIE	Molecular initiating event
NNF	Neural network formation
OECD	Organisation for Economic Co-operation and Development
PoD	Point of Departure
TTX	Tetrodotoxin
US EPA	United States Environmental Protection Agency
VGSCs	Voltage Gate Sodium Channels