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Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment

Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: Neurotoxicity and developmental neurotoxicity

Hazard Identification

Epidemiological studies

1. For the health outcome category (HOC) Neurotoxicity and developmental neurotoxicity, a total of 18 studies was appraised by the EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) Panel.

Identification of the clusters to be considered for Weight of Evidence

2. On the basis on the approach, the following Clusters (C) and Exposure periods (Exp) were brought forward to Weight of Evidence (WoE) analysis:
C: Neurodevelopment

Exp: Pregnancy and Childhood.

WoE of the relevant clusters

3. The main information extracted from the studies included in relevant clusters in the HOC Neurotoxicity and developmental neurotoxicity are summarised in Annex C of the EFSA re-evaluation (2021). The outcome of the weight of the evidence is described in the text below.

Cluster Neurodevelopment

Exposure during pregnancy and childhood

4. A large number of endpoints related to neurodevelopment were studied in the 18 publications pertaining to 14 longitudinal studies all assessing exposure during pregnancy (Braun *et al*., 2014; Evans *et al*., 2014; Casas *et al*., 2015; Roen *et al*., 2015; Perera *et al*., 2016; Braun *et al*., 2017a; Braun *et al*., 2017c; Braun et al., 2017d; Giesbrecht *et al*., 2017; Lim *et al*., 2017; Lin CC *et al*., 2017; Minatoya *et al*., 2017a; Philippat *et al*., 2017; Stacy *et al*., 2017; Ghassabian *et al*., 2018; Kim *et al*., 2018a; Minatoya *et al*., 2018; Nakiwala *et al*., 2018).

(Note: Braun et al., 2017c was considered as a cross-sectional study in the EFSA supporting publication 'Implementation of the evidence-based risk assessment for the re-evaluation of Bisphenol A: preparatory work on cross-sectional studies' (AERU, University of Hertfordshire, 2020) but was recategorized by the EFSA WG on BPA re-evaluation as a cohort study and therefore considered in the WoE assessment).

5. BPA exposure was measured via a single spot urine sample in all studies and varied between studies. The populations under study were of comparable sample size but varied in their characteristics; there were eight studies including a European-descent population. The observed heterogeneity for endpoint definitions was considerable including a large number of questionnaires evaluating cognition, behaviour, intellectual ability and psychomotor development. No statistically significant associations were observed in more than one study. Thus, the currently available longitudinal epidemiological evidence is characterised by a small number of studies, suboptimal exposure assessment and considerable heterogeneity in the assessed populations, exposure levels and endpoints. In summary, the currently available epidemiological evidence does not put forward an endpoint related to neurodevelopment as a critical one for risk assessment.

6. In the text below, the assessed longitudinal studies are described in brief.

7. The HOME study is a birth cohort conducted in the USA (Braun *et al*., 2017b). A total of pregnant women were enrolled between March 2003 and February 2006 in Cincinnati, Ohio, who delivered live singleton infants. Pre-natal exposure assessment was done at around 16 weeks of gestation. Post-natal exposure assessment was done at 1, 2, 3, 4 and 5 years of age. Median maternal urinary BPA concentrations during pregnancy were 2.0 mg/g creatinine (range: 0.4–49). Children were followed up at 1, 2, 3, 4 and 5 years of age for neurodevelopment, physical growth and health conditions. Three publications report on the results of various associations pertinent to the present EFSA Opinion. Braun *et al*. (2014a) investigated the association between pre-natal exposure to BPA (n = 175) and autistic behaviours at 4 and 5 years (Social Responsiveness Scale, SRS). No statistically significant association was observed. Braun *et al*. (2017d) assessed the association between pre-natal BPA exposure (16, 26 weeks) and neurodevelopment until 8 years old (n = 229). The included neurodevelopment endpoints pertained to behaviour (BASC-2), mental and psychomotor development (Bayley Scales of Infant Development-II, BSID-II) and child cognitive abilities (Wechsler primary and preschool scale of intelligence–III (WPPSI-III), Wechsler intelligence scale for children IV (WISC-IV)).

8. Overall, no statistically significant associations were observed. In girls, each 10-fold increase in maternal urinary BPA concentrations was associated with a 5.9-point increase (95% CI: 1.1, 11) in BASC-2 externalising scores and nearly 6-times the risk of having a score 60 (RR = 5.8; 95% CI: 1.7, 20). Finally, Braun *et al*. (2017a) assessed children's visual-spatial abilities at 8 years of age using the Virtual Morris Water Maze (VMWM), a computerised version of the rodent Morris water maze (MWM). Pre-natal BPA exposure was not associated with VMWM performance.

9. Braun *et al*. (2017c) in the MIREC birth cohort assessed the association between pre-natal BPA exposure at 12 weeks of gestation and child neurobehaviour at 3 years of age (n = 812; WPPSI- III, BRIEF-P, BASC-2, Social responsiveness scaleE–2/SRS-2). Overall, while BPA exposure was not associated with WPPSI-III scores, the BASC-2 or BRIEF-P scales, a statistically significant association was observed for the SRS-2 scores (b = 0:3; 95% CI: 0, 0.7).

10. The EDEN study (Philippat *et al*., 2017) is another birth cohort conducted in France between February 2003 and January 2006 (n = 529). BPA and 19 additional phthalate metabolites and 6968 phenols (four parabens, benzophenone-3, two dichlorophenols, triclosan) were measured in spot urine samples collected during pregnancy among mothers who delivered a boy. Behaviour was investigated using the Strength and Difficulties Questionnaire (SDQ) at 3 and 5 years of age. No overall statistically significant association was observed.

11. Nakiwala *et al*. (2018) assessed verbal and performance IQ at 5–6 years (WPPSI). No statistically significant association was observed. The Columbia Center for Children's Environmental Health (CCCEH, n = 727) is a birth cohort study recruiting African-American and Dominican pregnant women between 1998 and 2006 in USA. BPA was quantified in maternal urine collected during the third trimester of pregnancy and in child urine collected at ages 3 and 5 years.

12. Neurodevelopment was assessed periodically using the Revised Children's Manifest Anxiety Scale (RCMAS) and Children's Depression Rating Scale-Revised (CDRS) at 10–12 years. Two CCCEH publications are included in the present opinion. Roen *et al*. (2015) and Perera *et al*. (2016) included 239 children in their assessment and found no statistically significant associations between pre-natal BPA and the scales' overall score for depression and anxiety. Among the numerous analyses performed, statistically significant associations were found for boys for the overall score results.

13. Evans *et al*. (2014) in the SSF II study in USA evaluated pre-natal BPA exposure in relation to child behaviour at 6–10 years (Child Behaviour Checklist (CBCL), n = 153). No statistically significant association was observed.

14. Casas *et al*. (2015) reporting on the INMA birth cohort in Spain evaluated the association between pre-natal BPA and cognitive and psychomotor development at 1 and 4 years (Bayley Scales of Infant Development BSID, MCSA) and attention deficit hyperactivity disorder (ADHD) symptoms (ADHD-DSM-IV) and other behavioural problems (CPRS, SDQ) at 4 and 7 years. At 1 year of age, exposure in the highest BPA tertile was associated with a reduction of psychomotor scores

(BSID, T3 6992 vs T1, β = −4.28 points, 95% CI −8.15 to −0.41). At 4 years, BPA exposure was associated with an 6993 increased risk of ADHD hyperactivity symptoms (IRR per log10 BPA increase 1.72; 95% CI 1.08, 2.73) both overall and in boys. No statistically significant association was observed for the remaining endpoints.

15. Lim *et al*. (2017) in the Environment and Development of Children study in Korea evaluated pre-natal BPA exposure and neurobehaviour at 4 years (K-SCQ, n = 304). No statistically significant association was observed for the prospective component of the study.

16. Lin CC *et al*. (2017) in the TBP birth cohort in China assessed pre-natal BPA (cord blood, 7000 n = 208) in relation to neurodevelopment at 2 and 7 years (Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT), WISC-IV). In the WISC-IV neurocognitive assessment, a significant negative association was found for the overall scale as well as for various scale domains.

17. Minatoya *et al*. (2017a) in the Hokkaido Study on Environment and Children's Health Study in Japan investigated the association between pre-natal BPA levels (cord blood) and neurodevelopment up until 3.5 years (CBCL, K-ABC, BSID-II, n = 285). Although no overall statistically significant findings were reported among the numerous analyses performed, cord blood BPA concentration was statistically significantly positively associated only with CBCL development problems 7008 score (β = 2.60, 95% CI: 0.15, 5.06). In another publication of the same study, (Minatoya *et al*., 2018) examined the association between maternal (1st trimester) BPA exposure and behaviour at 5 years using the Strengths and Difficulties Questionnaire (SDQ) in 458 children. The median concentration of BPA was 0.062 ng/ml and no overall statistically significant findings were reported. Among the numerous analyses performed, BPA levels were associated with an increased risk of prosocial behaviour (OR = 1.46, 95% CI 1.04–2.06).

18. Kim *et al*. (2018a) in the Korean CHECK birth cohort (n = 140) investigated the association between four phthalates, BPA, three heavy metals, 19 polychlorinated biphenyls (PCBs), 19 organochlorine pesticides and 19

polybrominated diphenyl ethers, and early neurodevelopment (13–24 months of age). For the endpoint assessment the following tools were used: BSID-II, Social maturity scale (SMS) and CBCL. No statistically significant associations were observed for BPA in the overall cohort.

19. Ghassabian *et al*. (2018) in the Upstate KIDS birth cohort investigated the association between perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and BPA (Gathrie cards) and behaviour at age 7 (Strengths and Difficulties Questionnaire, n = 650 singletons to 138 twins). The median (interquartile range) of BPA was 7.93 ng/ml (10.79). No statistically significant association was observed for BPA and total behavioural difficulties (continuous and categorical analyses).

20. Giesbrecht *et al*. (2017) in the Alberta Pregnancy Outcomes and Nutrition study (birth cohort, n = 132) examined the association between pre-natal maternal urinary BPA concentration and cortisol and cortisol reactivity at age 3 months. The association between maternal total BPA concentration and baseline infant cortisol (β = 0.13, 95% CI: −0.01, 0.28) or cortisol reactivity (−18% decrease per hour for females per 10-fold increase in BPA, 95% CI: −35, 3) was not significant when infant sex and creatinine was considered in the model.

21. Four studies also assessed BPA exposure during childhood (Roen *et al*., 2015; Perera *et al*., 2016; Stacy *et al*., 2017; Kim *et al*., 2018a).

22. Perera *et al*. (2016) included 239 children in their assessment and found no statistically significant associations between post-natal BPA and the scales' overall score for depression and anxiety.

23. Roen *et al*. (2015) included 250 children in their assessment and found statistically significant associations between post-natal BPA and internalising and externalising scores on the CBCL. Girls and boys were respectively showing increasing and decreasing problems.

24. Stacy *et al*. (2017) in a HOME study publication attempted to extend these findings and to identify potential windows of vulnerability using repeated measures of pre-natal BPA exposures. Among all children, there was not strong evidence that the

associations between BPA and neurobehaviour varied by the timing of exposure (Visit × BPA p-values ≥ 0.16).

25. Finally, Kim *et al*. (2018a) in the Korean CHECK birth cohort investigated the association between post-natal BPA exposure via breast milk sampling at 30 days after delivery (n = 73) and early neurodevelopment (13–24 months of age; BSID-II, SMS, CBCL). No statistically significant associations were observed for BPA in the overall cohort.

Overall conclusions

26. On the basis of the above, the CEP Panel concluded that the evidence for an association between BPA exposure and impaired neurodevelopment is Not Likely.

Cross-sectional studies

27. Ten cross-sectional studies investigated BPA exposure and childhood behaviour, learning disabilities and autism (Findlay and Kohen, 2015; Stein *et al*., 2015; Arbuckle *et al*., 2016; Kardas *et al*., 2016; Kondolot *et al*., 2016; Perez-Lobato *et al*., 2016; Tewar *et al*., 2016; Rahbar *et al*., 2017; Li Y *et al*., 2018c; Metwally *et al*., 2018).

28. All but one (Rahbar *et al*., 2017) yielded statistically significant results pertaining to various clinical endpoints (autism, n = 3 (Stein *et al*., 2015; Kardas *et al*., 2016; Metwally *et al*., 2018); ADHD = 3 (Arbuckle *et al*., 2016; Tewar *et al*., 2016; Li Y *et al*., 2018c); Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS), n = 1 (Kondolot *et al*., 2016)) and scales ((SDQ), n = 2 (Findlay and Kohen, 2015; Arbuckle *et al*., 2016); CBCL, n = 1 (Perez-Lobato *et al*., 2016)) and across different populations and studies of varying power. The available cross-sectional evidence is not aligned with the available longitudinal evidence where most associations neither reached statistical significance, nor were replicated by subsequent research.

Animal studies

29. For the HOC Neurotoxicity and developmental neurotoxicity a total of 94 studies was appraised by the CEP Panel. The details of the appraisals (internal and external validity) are reported in Annex E of the EFSA re-evaluation (2021).

30. The endpoints for each study identified as relevant in this opinion are reported in Annex F of the EFSA re-evaluation (2021). Effects on behaviour were already key endpoints in the uncertainty analysis in the 2015 EFSA opinion (EFSA CEF Panel, 2015).

Identification of clusters of relevant endpoints

31. Endpoints for which statistically significant changes were reported were extracted from the available literature and grouped into several clusters related to brain morphology and function. These clusters have been evaluated in a WoE approach and the results are described here.

32. Rather than identifying all the multiple pathways these endpoints can interact with, the CEP Panel decided to group them into three empirical clusters that represent different aspects of brain function: neuromorphology, nervous system functionality and behaviour. However, it is important to note that they are more or less interrelated and are not independent of each other; for example, dendritic spine plasticity as a driver or consequence of behaviour (Gipson and Olive, 2017). They all contribute to perception, cognition and integrated responses that enable an organism to cope with its changing environment.

33. Potential connections between these clusters will be explored in the section on hazard identification:

1) Neuromorphology (including neuronal dendrite morphology, dendritic spine density (hippocampus, neocortex), brain nucleus size/volume, number of neurons/glia in various brain regions).

2) Nervous system functionality (neurochemistry, electrophysiology, brain regional neurotransmitter/receptor/hormone content, neuronal responsiveness).

3) Behaviour (anxiety-related behaviour, learning and memory, social behaviour, taste preference, locomotor activity, sensory/motor coordination) as the most apical outcome of brain function.

Table 1. Clusters of relevant endpoints

Clusters	Relevant Points
Neuromorphology	Dendrite branching Dendrite intersections Dendrite length Hypothalamic histology (kisspeptin-ir cells in anteroventral periventricular nucleus (AVPV), rostral periventricular area (rPen), caudal periventricular nucleus (cPen)) Number of dopamine (DA) neurons in neocortex Number of DA neurons in ventral mesencephalon (VM) Number of glia within medial prefrontal cortex (mPFC) Pro-opiomelanocortin (POMC) projection into the paraventricular nucleus (PVN) POMC projections to hypothalamus Dendritic spine density in CA1 pyramidal cells Dendritic spine density in in hippocampus Dendritic spine density in CA1 region of hippocampus Dendritic spine synapses in CA1 region

	Dendritic spine density in hippocampal dentate gyrus Dendritic spine head size of dentate gyrus neurons Dendrite length (hippocampus) Number of hippocampal CA1 neurons Number of hippocampal CA3 neurons Dendritic spine density on CA1 pyramidal cells Dendritic spine density in CA1 regions Dendritic spine density on layer II/III on mPFC pyramidal cells Dendritic spine density in layers 2/3 of dorsolateral prefrontal cortex (DLPFC) Hypothalamic histology (number of kisspeptin-ir cells in AVPV) Spine synapses in CA1 regions Spine synapses in layers 2/3 of DLPFC
Nervous system functionality	Gamma amino butyric acid (GABA) (hippocampus) GABA (cortex) Aspartate (hippocampus) Aspartate (cortex) Glutamine (GLN) (hippocampus) GLN (cortex) Noradrenaline (NA) (hippocampus) Dopamine and metabolite (hippocampus) Serotonin (hippocampus) Oxytocin receptor (OTR) density (in posterior bed nucleus of stria terminalis (BNSTp), ventromedial hypothalamus

	(VMH), PVN and dorsolateral bed nucleus of the stria terminalis (BNSTdl)) Excitatory and inhibitory responsiveness of recorded medial amygdala neurons to neurochemical signals Leptin blood concentration Leptin sensitivity Serum corticosterone Corticosterone production Acetylcholinesterase (AChE) activity (in prefrontal cortex, hypothalamus, cerebellum and hippocampus) GLU (cortex) GLU (hippocampus) Glycine (GLY) (cortex) GLY (hippocampus) Monoamino-oxidase (MAO) activity Serum corticosterone Taurine (cortex) Taurine (hippocampus)
Behaviour	Anxiety/emotionality (avoidance of predator odour, elevated plus maze (EPM), elevated zero maze (EZM), forced swimming test (FST), open field test (OFT), dark light test (DLT)) Learning and memory (radial arm maze (RAM), reference memory, Barnes maze, Morris water maze (MWM), object recognition, fixed interval reinforcement, object placement, Y maze; working memory, spatial memory)

	Locomotor activity (exploratory behaviour, open field test (OFT), running activity, spontaneous activity, hole poke test, tremor activity (electronic balance test), EPM, EZM, mirrored maze) Preference behaviour (sodium salt intake; sweet preference; water intake) Social behaviour (maternal behaviour; female sexual behaviour; male sexual behaviour; interaction) Sensory-motor coordination (rotarod experiment; string test)

Source: Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA, (2021)

Neuromorphology

34. The first cluster comprises endpoints related to brain development, including effects on neurogenesis and on the morphology of the various brain regions. It includes the number of cells, the dendritic spine density and the degree of connectivity between cells, as well as brain volume and growth. Such relevant endpoints can be considered at the level of the whole brain or a specific brain area.

Nervous system functionality

35. The second cluster considers endpoints related to brain function, including several systems of neurotransmission (GLU, GABA, serotonin, noradrenaline, dopamine) within the brain and hormone communication (noradrenaline, steroid and peptide hormones) with the whole body. Electrophysiological neuronal responsiveness is also considered within this cluster as neurons propagate chemical signals by generating electric potentials.

Behaviour

36. The third cluster comprises behavioural endpoints including anxiety-related behaviour, learning and memory, social behaviour and sensory-motor function, which were considered by EFSA in 2015 (EFSA CEF Panel, 2015) to be as likely as not (ALAN) related to BPA exposure.

37. Behaviour represents the highest level of integrated function between the brain, the organism and the environment. Behavioural disturbances may cause problems for human health and be indicative of neurodevelopmental syndromes such as attention deficits, autism, schizophrenia and neurodegenerative processes later in life such as dementia, Alzheimer's or Parkinson's disease. However, even subtle effects can potentially impact all stages of human life from birth to old age, starting with mother-child bonding and parenting, the education process and the interaction with peers.

38. For adversity to human, endpoints of the clusters Neuromorphology and Nervous system functionality are highly interrelated, representing anatomical signalling pathways, and signal generation and transduction, respectively.

39. Depending on the study, endpoints of the clusters of Neuromorphology and Nervous system functionality were studied at the level of the whole brain or a specific brain region. A neurofunctional or neuroanatomical effects with the same causal mechanism but occurring in different parts of the brain may have different outcomes depending on the brain region. As a consequence, measurement done at the level of the whole brain is less indicative of specific BPA-related changes. In addition, effects observed in any of the three clusters may not be identical for males and females due to the known sex- specific development of the brain. These gender-related aspects of brain function may necessitate separate evaluations in males and females to determine if sex could influence the neurotoxicity of BPA.

40. Finally, brain weight, which is a general endpoint used in neurotoxicity, does not seem to be specifically relevant due to a lack of significant effects of BPA in any of the reported studies. The lack of significant effects could be explained by the fact

that the brain is a well-protected organ against most insults and the brain weight is an endpoint that remains very difficult to disrupt.

WoE of the clusters of relevant endpoints

41. The main information extracted from the studies addressing relevant endpoints in the HOC Neurotoxicity and developmental neurotoxicity are summarised in Annex G of the EFSA re-evaluation (2021).The outcome of the WoE is described in the text below and presented in a tabulated format in Annex H of the EFSA re-evaluation (2021). The clusters of the effects of BPA on Neurotoxicity and developmental neurotoxicity considered for this assessment were the following:

- Neuromorphology
- Nervous system functionality
- Behaviour

Neuromorphology

42. The cluster Neuromorphology includes various endpoints which relate to different types of actions or responses that can be observed in animals or humans. For the sake of clarity, the results of the WoE exercise will be presented by endpoint to demonstrate which neuromorphological measurements were found to be affected by BPA exposure of a certain age group and which were not.

43. The specific measurements that were included for the effects of BPA on neuromorphology concern the cell number and/or volume (mostly concerning dopaminergic neurons), the dendritic morphology and/or density, and the number of spine synapses. It has to be noticed that most studies report such measurements in various parts of the brain, cover more than one endpoint and assess such endpoints relatively to specific behavioural performances.

44. The complete cluster Neuromorphology includes 15 studies, eight being conducted in rats, six in mice, one in rhesus monkeys and one in vervet monkeys. Animals were exposed during development in seven studies (two in rats, four in mice and one in rhesus monkeys) or during the growth phase/young age in six studies (four in rats, one in mice and one in rhesus monkeys). Two studies were conducted at the adult stage, one in mice and one in vervet monkeys.

Developmental exposure (pre-natal and/or post-natal until weaning)

45. Seven studies were identified concerning this period of exposure. Two studies were performed in rats, four in mice, and one in rhesus monkeys (*Macaca mulatta*). One of the rat studies was allocated to Tier 2 (Liu ZH *et al*., 2014) and one to Tier 3 (Liu ZH *et al*., 2015). In mice, two studies were allocated to Tier 1 (Komada *et al*., 2014; MacKay *et al*., 2017) and two to Tier 2 (Kimura *et al*., 2016; Naule *et al*., 2014). The study performed in rhesus in monkeys was allocated to Tier 1 (Elsworth *et al*., 2013).

46. Four studies reported effects of BPA on dendritic spine density measured in two highly plastic parts of the brain, namely hippocampus and pre frontal cortex (PFC). The Tier 1 study from Elsworth *et al*. (2013) reported that a gestational exposure to BPA for 50 days using subcutaneous implants in rhesus monkeys (*Macaca mulatta*), delivering an estimated level of exposure of 550 µg/kg bw per day, was associated with a significant loss of spine synapses in CA1 hippocampus, but not in the dorsolateral part of PFC. Two Tier 2 studies revealed similar results in the same brain region, one in mice (Kimura *et al*., 2016) and one in rats (Liu ZH *et al*., 2014). In mice (Kimura *et al*., 2016) BPA exposure from GD8.5 to GD18.5 at the highest dose of 400 µg/kg bw per day induced a significant reduction in the three different endpoints measured at PND21, all related to the synaptic connectivity in the hippocampal CA1 area. These endpoints are the number of 5th order branching, the number of intersections >40 µm from cell body and the dendrite length. No changes were observed at the lowest dose level (40 µg/kg bw per day). Both reductions were observed only in basal dendrites at the dose of 400 µg/kg bw per day, whereas the apical part of dendrites remained unaffected at any dose. Only males were studied.

In rats (Liu ZH *et al*., 2014) a 7-day post-natal exposure from PND7 to PND14 induced a significant BPA dose-related decrease in the dendritic spine density of dentate gyrus neurons (−5% at 50 µg/kg bw per day, −12% at 250 µg/kg bw per day and −15% at the dose of 500 µg/kg bw per day). A concomitant 25% reduction in the dendritic spine head size of the same neurons was observed in the same region at all three dose levels.

47. The Tier 3 study from Liu ZH *et al*. (2015) revealed a dose-related reduction in the dendritic spine density of CA1 hippocampal neurons (0%, −4% and −13%) in 12-week-old rats post- natally exposed to BPA (50, 250 or 500 µg/kg bw per day from PND7 to PND14, *i.p.* administration). A more important reduction has been measured in the same study in 12-week-old rats after chronic exposure to BPA via oral maternal dosing (GD0–PND21) and post-natal dosing (PND21–12 weeks of age) through drinking water. Two levels of exposure were applied (0.15 and 7.5 µg/kg bw per day) leading to 14% and 21% decreases in the dendritic spine density, respectively. Only males were tested in both experiments. This study is reported here despite its allocation to Tier 3 because it revealed comparable results related to the same endpoints as reported in other Tier 1 or Tier 2 studies (Elsworth *et al*., 2013; Kimura *et al*., 2016; Liu ZH *et al*., 2014) and then highly contributes to the final likelihood per endpoint (Likely) and per cluster (Likely).The effect of BPA on the dendritic spine density in the same brain region in three different species was judged as Likely.

48. A Tier 3 study conducted in rats (Sadowski *et al*., 2014b) reported a significant increase in the number of neurons (+15%) and glial cells (+19%) in layers 5–6 of the medial PFC at PND140 only in males early exposed to the highest dose of BPA (GD0–PND9, 400 µg/kg bw per day). No effects were observed in males at the low and mid doses studied (4 and 40 µg/kg bw per day) or in females at any dose. No effect was observed in layers 2–3 of the same part of the brain in males and females. This Tier 3 study is the only one available exploring the number of glial cells in medial PFC. The evidence was considered Inadequate to conclude on the likelihood of the effect.

49. Two studies investigated the plasticity of the dopaminergic system in various parts of the brain, one in mice (Komada *et al*., 2014) and one in monkeys (Elsworth *et al*., 2013).

50. The Tier 1 study (Komada *et al*., 2014) was performed in mice and showed at PND3 a significant reduction in the projection of dopaminergic neurons in the neocortex (from layer 1 to 6) of pre-natally BPA-exposed newborns at the two doses tested. BPA was administered orally from GD6 to GD18 at a dose of 20 or 200 µg/kg bw per day. Effects of BPA on this endpoint was judged as ALAN for concluding on the likelihood of the BPA effects.

51. The other Tier 1 study related to the dopaminergic system (Elsworth *et al*., 2013) investigated the effects of a single-dose of BPA on the same endpoint considered by Komada *et al*. (2014) but in rhesus monkeys (*Macaca mulatta*). Results showed a significant reduction in the number of dopaminergic TH-immunoreactive neurons in VM, a part of the brain including dopaminergic regions like substantia nigra and ventral tegmental area, in newborn monkeys gestationally exposed to BPA (400 µg/kg bw per day, GD100–GD165, oral administration by food). The evidence was considered Inadequate to conclude on the likelihood of the effect.

52. The Tier 2 study from Naule *et al*. (2014) investigated the number of kisspeptin-immunoreactive cells in three brain areas, the AVPV and the rostral and caudal parts of the periventricular nucleus. Results showed a significant increase in the number of kisspeptin- immunoreactive cells at both doses of BPA 50 and 5000 µg/kg bw per day (+25% and +35%, respectively) limited to the rostral part of the periventricular nucleus. This endpoint was judged as ALAN for concluding on the likelihood of the BPA effects.

53. A Tier 1 study (MacKay *et al*., 2017) assessed the effects of a perinatal exposure to BPA (3 µg/kg bw per day through the diet) from GD0 to PND21 on hypothalamic feeding circuitry in mice pups. At PND21, BPA induced a significant reduction in density of POMC immunolabeled fibres in the periventricular nucleus in males and females whereas the counting of POMC neurons in the arcuate nucleus

was unchanged in both sexes, suggesting that BPA did not alter the embryonic neurogenesis of POMC neurons. The evidence was considered to be Inadequate to conclude on the likelihood of the effect.

54. The CEP Panel assigned a likelihood level of Likely to the Neuromorphology effect of BPA in the developmental exposure period, based on Likely effects for dendritic spine density (Kimura *et al*., 2016; Liu ZH *et al*., 2014). Therefore, these studies were taken forward for BMD analysis. Moreover, the Likely and ALAN endpoints were also considered in the uncertainty analysis.

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

55. No studies were available for this exposure period.

Growth phase/young age exposure

56. Six studies were identified in this exposure period, all of them allocated to Tier 1. Four studies were conducted in rats (Chen Z *et al*., 2018; Wise *et al*., 2016; Bowman *et al*., 2014; Bowman *et al*., 2015, one in mice (Zhou YX *et al*., 2017) and one in vervet monkeys (African green monkeys, *Chlorocebus aethiops sabaeus*) (Elsworth *et al*., 2013).

57. These studies explored the neuro-morphological effects of BPA in three different brain regions, namely hippocampus, PFC and mesencephalon.

58. In hippocampus, the Tier 1 study from Zhou YX *et al*. (2017) revealed a reduced number of neurons in hippocampal CA1 and CA3 areas) of juvenile male rats (PND56) exposed orally to BPA for 8 weeks from birth to post-natal week (PNW) 8 at three doses, 0.5, 50 and 5000 µg/kg bw per day. In the CA1 area, the number of neurons was significantly lower (about 10%) compared with controls at the highest dose of BPA (5000 µg/kg bw per day). In the CA3 area, the number of neurons was significantly lower than in controls (also about 10%) at 0.5 and 5000 µg/kg bw per

day. Only males were tested. The evidence for these endpoints was judged as Likely.

59. A Tier 1 study in rats (Chen Z *et al*., 2018) reported a decrease in the hippocampal CA1 dendritic spine density in animals exposed to 40, 400 and 4000 µg/kg bw per day of BPA after weaning (PND21–PND49) but it was significant only at the highest dose compared with controls. No effect of BPA on the dendritic length was observed in the same region. Only males were tested.

60. Two Tier 1 studies (Bowman *et al*., 2014; Bowman *et al*., 2015) investigated the dendritic spine density of hippocampal CA1 pyramidal neurons in both basal and apical parts of the cell at two post-natal ages (PND49 or PND91) in rats exposed subcutaneously to 40 µg/kg bw per day of BPA, from PND42 to PND49. At PND49, spine density was lower in the BPA group in both basal (−24%) and apical (−15%) parts of the CA1 pyramidal cells (Bowman *et al*., 2014). Males, both controls and BPA-treated animals, had fewer spines in CA1 basal dendrites (−9%) than control and BPA-exposed females, with no significant interaction between treatment and sex. At adulthood (PND91), a reduction in basal and apical CA1 dendritic spine densities was observed in both males and females compared with control animals (−19% and −21%, respectively). No significant interaction between treatment and sex was noted. Considering the above evidence, the endpoint dendritic spine density of pyramidal cells in hippocampal CA1 area was judged as Likely.

61. The Tier 1 study of Elsworth *et al*. (2013), reported no difference in the hippocampal CA1 dendric spine density regions measured at the end of dosing in vervet monkeys (African green monkeys, *Chlorocebus aethiops sabaeus*, 14–18 months of age) continuously exposed to BPA (550 µg/kg bw per day) through subcutaneous implants for 30 days during the pre-pubertal period. The evidence was considered to be Inadequate to conclude on the likelihood of the effect.

62. Regarding the PFC, the Tier 1 study from Wise *et al*. (2016) explored the total number of glial cells in the medial part of the PFC in both adult male and female rats (PND150) exposed during the brain growth phase (PND27–PND46) to BPA at a dose of 4, 40 or 400 µg/kg bw per day. Results showed similar increases in the

number of glial cells in females, rather microglial cells than astrocytes, and similar decreases in males. However, such differences were statistically significant referred to controls only at the dose of 40 µg/kg bw per day in females and 4 µg/kg bw per day in males. No effects on the number of neurons were observed at any dose in males and females. This endpoint was judged ALAN for the number of glial cells.

63. The two Tier 1 studies from Bowman *et al*. (2014) and Bowman *et al*. (2015) investigated at two post-natal ages (PND77 or PND91) the dendritic spine density of pyramidal neurons (basal and apical parts of the cell) in the medial part of PFC of rats exposed subcutaneously to 40 µg/kg bw per day of BPA from PND42 to PND49. At PND77, the BPA group had lower spine density in both basal (−21%) and apical (−10%) parts of the prefrontal pyramidal cells (Bowman *et al*., 2014). Both sexes showed the same effect on this endpoint in this part of the brain. At adulthood (PND91), no effect on basal and apical dendritic spine densities was observed in either males or females. The endpoint dendritic spine density of pyramidal cells in PFC was judged as Likely.

64. The Tier 1 single-dose study of Elsworth *et al*. (2013) reported no difference in the prefrontal dendric spine synapses measured in the cortical layers 2 and 3 at the end of the period of exposure in vervet monkeys (African green monkeys, *Chlorocebus aethiops sabaeus*, 14–18 months of age) continuously exposed to BPA through subcutaneous implants for 30 days during the pre-pubertal period (plasma levels 13.1 ± 1.4 ng/mL at termination after 30 days exposure; equivalent to 4500 µg/kg bw per day oral). The same study reported also the same absence of effect of BPA on the number of dopaminergic neurons measured in the VM of the same animals.

65. The evidence for dendritic spine density of pyramidal cells in PFC and the number of dopaminergic neurons in mesencephalon was judged Inadequate to conclude on the likelihood of the effect.

66. Overall, the CEP Panel assigned a likelihood level of Likely to the Neuromorphology effects of BPA in the growth phase/young age exposure period. Since the likelihood for Neuromorphology is Likely for the endpoints Number of

hippocampal CA1 neurons (Zhou YX *et al*., 2017), Number of hippocampal CA3 neurons (Zhou YX *et al*., 2017) and dendritic spine density on CA1 pyramidal cells (Chen Z *et al*., 2018), these studies were taken forward for BMD analysis. The endpoint dendritic spine density on layer II/III pyramidal cells in the PFC, also Likely, was not taken forward for BMD analysis because only single-dose studies were identified for this endpoint. However, the Likely and ALAN endpoints were considered in the uncertainty analysis.

Adult exposure (after puberty)

67. Only two studies from two different species were identified in this exposure period, one in mice and one in vervet monkeys.

68. The study performed in mice (Wang XL *et al*., 2014) was allocated to Tier 2 and reported a lack of changes in the number of kisspeptin-immunoreactive cells in the anteroventral part of the periventricular nucleus of females 6 hours after a unique oral administration of 20 µg/kg bw per day of BPA. The second study (Elsworth *et al*., 2015) is a

69. Tier 1 study performed in adult male vervet monkeys (*Chlorocebus sabaeus*) that consisted in administrating BPA subcutaneously using an osmotic minipump to achieve a dose of 50 µg/kg bw per day for 30 days (this level of dose was calculated to correspond to an equivalent oral dose of 5556 µg/kg bw per day). The results showed a significant reduction in the number of spine synapses measured at the end of exposure in hippocampus (CA1 stratum radiatum) and PFC (layers 2/3 of the dorsolateral part of PFC) compared with controls. It is noted that measurements done 4 weeks after the removal of the minipump and the end of BPA exposure showed a partial recovery of the number of synapses into the same regions.

70. These studies were considered to be of Inadequate evidence for the endpoint related to the hypothalamic histology (Wang XL *et al*., 2014) and for the endpoints number of spine synapses in hippocampus and PFC (Elsworth *et al*., 2015).

71. The CEP Panel considered the evidence Inadequate to assign a likelihood level to the Neuromorphology effects of BPA in the adult exposure period. Therefore, none of the endpoints was taken forward for BMD analysis.

Indirect (germline) exposure

72. No studies were available for this exposure period.

Overall cluster selection of the endpoints/studies for BMD analysis for Neuromorphology

73. Overall, the CEP Panel assigned a likelihood level of 'Likely' to the effects of BPA on neuromorphology in the exposure periods developmental (pre-natal and/or post-natal until weaning) and growth phase/young age, and Inadequate evidence in the period adult exposure (after puberty).

74. The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period developmental (pre-natal and/or post-natal until weaning) for the endpoint 'Dendritic spine density of pyramidal cells in hippocampus (CA1 and dentate gyrus areas)' (Kimura *et al*., 2016; Liu ZH *et al*., 2015; Elsworth *et al*., 2013; Liu ZH *et al*., 2014). The endpoint 'Dendritic spine density of pyramidal cells in hippocampus (CA1 and dentate gyrus areas)' (Kimura *et al*., 2016; Liu ZH *et al*., 2014c) was taken forward for BMD analysis. The same endpoint assessed in the studies by Liu ZH *et al*. (2015) and Elsworth *et al*. (2013) was not taken forward because the first is a Tier 3 study and the second is a single-dose Tier 1 study.

75. The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period Growth phase/young age for the endpoints 'Number of neurons in hippocampus (CA1 and CA3 areas)' (Zhou YX *et al*., 2017), 'Dendritic spine density in CA1 pyramidal cells' (Bowman *et al*., 2014; Bowman *et al*., 2015; Chen Z *et al*., 2018), and 'Dendritic spine density in pyramidal cells in medial part of PFC' (Bowman *et al*., 2014; Bowman *et al*., 2015).

76. The endpoints 'Number of neurons in hippocampal CA1 area' (Zhou YX *et al*., 2017), 'Number of neurons in hippocampal CA3 area' (Zhou YX *et al*., 2017b) and 'Dendritic spine density in CA1 pyramidal cells' (Chen Z *et al*., 2018) were taken forward for BMD analysis.

77. Both endpoints 'Dendritic spine density in CA1 pyramidal cells' and 'Dendritic spine density in pyramidal cells in medial part of PFC (Bowman *et al*., 2014; Bowman *et al*., 2015 were not taken forward for BMD analysis because these two Tier 1 studies are both single-dose studies.

78. The CEP Panel assigned a likelihood level of Inadequate evidence to the effects of BPA on neuromorphology in the exposure period 'adult exposure (after puberty)'. Therefore, none of the endpoints was taken forward for BMD analysis.

79. The overall likelihood across all exposure periods, *i.e.* the highest likelihood given in the cluster Neuromorphology, was Likely.

Nervous system functionality

80. As for the cluster Neuromorphology, this cluster includes various endpoints that relate to different types of actions or responses that can be observed in animals or humans. For the sake of clarity, the results of the WoE exercise will be presented by endpoint to demonstrate which brain functioning measurements were found to be affected by BPA exposure of a certain age group and which were not. The specific measurements that were included for the effects of BPA on brain functionality concern various neurotransmitter systems (GABA, GLU, aspartate (ASP), TAU, GLY, monoaminergic systems), AChE activity, leptin secretion and corticosterone secretion and regulation. Most of these studies report such measurements in various parts of the brain, cover more than one endpoint and assess such endpoints relative to specific behavioural performances.

81. The complete cluster Nervous system functionality includes 10 studies, six in rats and four in mice. Animals were exposed during development in four studies (two

in rats, two in mice) or during the growth phase/young age in two studies (one in rats and one in mice). Four studies were performed in adult animals (one in mice and three in rats). Two studies in rats exposed the tested animals by indirect germline exposure.

Developmental exposure (pre-natal and/or post-natal until weaning)

82. Four studies in two animal species were identified for this part, two in mice (Xin *et al*., 2018; MacKay *et al*., 2017) and two in rats (Witchey *et al*., 2019; Fujimoto and Aou, 2018).

83. The Tier 2 study from Xin *et al*. (2018) investigated the brain levels of various neurotransmitters in the hippocampus of adult mice (21 weeks after birth) born from dams exposed to BPA from preconception (2 weeks before mating) until weaning (PND21) through the diet at two doses (10 and 10000 µg/kg bw per day). Results showed a significant dose-dependent decrease in serotonin (5HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in high-dose BPA-exposed adult male, while females were unaffected. Levels of noradrenaline (NA) were sex-specifically affected by BPA with a significant reduction in noradrenaline in low-dose BPA-exposed females. Dopamine (DA) levels were not affected in either sex at the low dose (10 µg/kg bw per day). A reduced level of the dopamine metabolite homovanillic acid (HVA) was also observed in low-dose BPA-exposed females. GABA, noradrenaline, dopamine and its metabolite and serotonin are endpoints which were judged as ALAN whereas the others (ASP, GLU and GLN) were judged as Not Likely.

84. The Tier 1 electrophysiological study from Fujimoto and Aou (2018) investigated the extracellular responses of neurons in the medial amygdala to the presentation of three plant odours and three predator odourants in adult male rats exposed to BPA during early development (GD0–PND14, 15 µg/kg bw per day through drinking water). Results showed a greater activity of amygdala odour-responsive neurons to two odourants (fox odour and whisky lactone) in BPA-exposed rats compared with the control animals. Females were not tested.

85. This study was considered to be Inadequate evidence for the BPA brain risk assessment for the endpoint of excitatory and inhibitory responsiveness of recorded medial amygdala neurons to neurochemical signals.

86. A Tier 1 study (Witchey *et al*., 2019) revealed significant sexually dimorphic differences in the density of oxytocin receptors (OTR) between control male and female rats aged 28 days after birth in three hypothalamic regions [Bed Nucleus of Striatal Terminalis (BNST), Paraventricular Nucleus (PVN) and Ventromedial Hypothalamus (VMH)]. In rats of the same age (PND28) exposed to BPA from GD6 to PND21 at 2.5, 25 or 2500 µg/kg bw per day, OTR binding was significantly increased compared with controls at 2.5 and 25 µg/kg bw per day in males only in the BNSTdl, which eliminated the sex- specific difference observed in the control group. No significant main effects of BPA exposure were measured in any other hypothalamic areas (BNSTp, PVN and VMH). No effects of BPA were found in females. This endpoint was judged as ALAN for concluding on the likelihood of the BPA effects.

87. The Tier 1 study from MacKay *et al*. (2017) revealed a delayed serum leptin surge from PND8 in control pups to PND10–12 in BPA-exposed rats. Exposure to BPA was performed in dams from GD0 to GD12 and in the offspring up to PND21 at a dose of 3 µg/kg bw per day. In addition, results showed a decrease in leptin sensitivity reflected by a lack of reduction in body weight in adult (PND130) BPA early exposed females and males after two days of leptin administration. Females were more strongly affected compared with the males.

88. This evidence was considered to be Inadequate for concluding on the likelihood of the BPA effect son the endpoint leptin (sensitivity and blood concentration).

89. The CEP Panel assigned a likelihood level of ALAN to the neurofunctional effects of BPA in the developmental exposure period, therefore none of the endpoints was taken forward for BMD analysis. However, the ALAN endpoints were considered in the uncertainty analysis.

Developmental and adult exposure (pre-natal and/or post-natal in pups until adulthood)

90. No studies were available for this exposure period.

Growth phase/young age

91. Two studies were identified during this exposure period. One was performed in mice (Luo *et al*., 2013), while the other one was done in rats (Bowman *et al*., 2015).

92. The Tier 1 study from Luo *et al*. (2013) reported a significant decrease in hippocampal AChE activity in young adult male rats immediately after the end of the period of exposure to BPA (PND30 to PND70; 10000 µg/kg bw per day) whereas the enzyme activity remained unchanged in the other brain regions studied (PFC, hypothalamus, cerebellum). Females were not tested. The evidence was considered to be Inadequate to conclude on the likelihood of the BPA effect on the endpoint 'AChE activity'.

93. The second study is also a Tier 1 study (Bowman *et al*., 2015) which reported a slight non- significant increase in corticosterone blood levels in male (+24%) and female (+16%) adult rats (PND91) exposed to BPA from PND42 to PND49 (40 µg/kg bw per day, s.c. equivalent to an oral dose of 1428 µg/kg bw per day) in a model of restraint-induced stress (1 hour, 21°C). The evidence was considered to be Inadequate to conclude on the likelihood of the effect.

94. The CEP Panel assigned a likelihood level of Inadequate evidence to the neurofunctional effects of BPA in the growth phase/young age exposure period, so, none of the endpoints was taken forward for BMD analysis.

Adult exposure (after puberty)

95. Four studies in two animal species were identified for this exposure period, one in mice (Khan *et al*., 2018) and three in rats (Fan *et al*., 2013; Khadrawy *et al*., 2016; Fan *et al*., 2018).

96. The study from Khan *et al*. (2018), which was allocated to Tier 2, reported a concomitant 30% decrease in AChE activity and significant increase (+40%) in monoamine oxidase (MAO) activity in the brain homogenate of adult male mice exposed to BPA at a dose of 10000 µg/kg per day (PND63–PND91). Only males were tested. The evidence was considered Inadequate for the endpoint MAO and as Likely for the other endpoint, AChE activity.

97. A Tier 1 study (Fan *et al*., 2013) reported a significant reduction of AChE activity measured in hippocampus of adult male rats at 144 days after birth and exposed to BPA 50 µg/kg per day for 10 weeks. Females were not considered for this study. The evidence for this endpoint was judged as Likely.

98. The Tier 2 study from Khadrawy *et al*. (2016) intend to assess the effects of BPA on AChE activity and excitatory (GLN, GLU and aspartate (ASP)) and inhibitory (GABA, GLY and taurine (TAU)) amino acid neurotransmitter levels in the cortex and hippocampus of adult male rats. Females were not tested. Two protocols of exposure to BPA were used: (1) BPA administered at two levels of doses, 10000 and 25000 µg/kg bw per day, for 6 weeks, and (2) BPA administered at only one level of dose, 10000 µg/kg bw per day, with two exposure times, 6 or 10 weeks. The results related to the AChE activity measured in the cortex and hippocampus revealed a significant dose-dependent and duration-dependent increase in the enzyme activity in both regions. Significant increases were observed in the cortex whatever the treatment regimen used. In the hippocampus, AChE activity was significantly increased only at the two dose levels administered for 6 weeks.

99. There were concomitant increases in hippocampal levels of both excitatory (GLN, GLU and ASP) and inhibitory (GABA, GLY and TAU) amino acid neurotransmitters with the two levels of doses given during 6 weeks. In the cortex,

the level of the GLU and ASP metabolic precursor GLN was reduced regardless of the dose or duration used for exposure. Cortical GLU and ASP levels were increased in a significant way only in animals exposed to 10000 µg/kg bw per day for 10 weeks, or to 25000 µg/kg bw per day for 6 weeks. Cortical GABA and GLY were significantly decreased in the same exposure groups. Finally, TAU was decreased in a significant way only in rats exposed to 25000 µg/kg bw per day of BPA during 6 weeks. The endpoint AChE activity was judged as Likely while the other ones (excitatory and inhibitory amino acid neurotransmitters) were judged as ALAN.

100. A Tier 1 study performed in adult male rats (Fan *et al*., 2018) dosed 0 or 50 µg/kg bw per day for 21 weeks through the diet) reported a significant increase in corticosterone blood levels 30 minutes after being tested in the open field compared with the basal level measured before the open field. The highest variation was observed in BPA-exposed rats compared with controls. Females were not included in this study. The evidence on serum corticosterone was judged Inadequate to conclude on the likelihood of the effect.

101. The CEP Panel assigned a likelihood level of Likely to the Neurofunctional adverse effect of BPA in the adult exposure period. The likelihood level for this cluster is Likely for the endpoint ‘AChE activity’ based on Khadrawy *et al*. (2016), therefore this endpoint was taken forward for BMD analysis.

102. Other endpoints related to this period of exposure, namely ‘excitatory or inhibitory neurotransmitters (GABA, ASP, GLU, GLN, GLY and TAU)’ (Khadrawy *et al*., 2016), ‘MAO activity’ (Khan *et al*., 2018) and ‘blood corticosterone production’ (Fan *et al*., 2018) were not considered for BMD analysis due to a likelihood level of ALAN based on a Tier 2 study (Khadrawy *et al*., 2016) or considered of Inadequate Evidence since only two single-dose Tier 1 or Tier 2 studies (Fan *et al*., 2018 and Khan *et al*., 2018, respectively) were available.

103. The Likely and ALAN endpoints were considered in the uncertainty analysis.

Indirect (germline) exposure

104. Two Tier 1 studies both performed in rats were identified (Fan *et al*., 2018; Fan *et al*., 2013) regarding this period of exposure.

105. The Tier 1 study from Fan *et al*. (2018) reported a significant increase (+25%) in corticosterone blood levels measured in adult female rats (PND56) derived from male parents exposed to BPA 50 µg/kg bw per day for 21 weeks before mating. Blood was examined 30 minutes after the FST which was the last one of a series of three consecutive behavioural tests including the Open Field, the EPM and the Forced Swim Test. No effect was observed in males.

106. This evidence was considered to be Inadequate to conclude on the likelihood of the effect The second Tier 1 study (Fan *et al*., 2013) reported a lack of significant changes in the hippocampal AChE activity measured in adult F1 male and female rats (PND56) issued from non-exposed F0 females being mated with F0 males exposed to BPA (50 µg/kg bw per day) for 10 weeks before mating.

107. This evidence was considered to be Inadequate to conclude on the likelihood of the effect. The CEP Panel assigned a likelihood level of Inadequate Evidence to the neurofunctional effects of BPA in the indirect (germline) exposure period, so, none of the endpoints was taken forward for BMD analysis.

Overall cluster selection of the endpoints/studies for BMD analysis for Nervous system functionality

108. The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the adult exposure (after puberty) period for the endpoint ‘AChE activity’ (Fan *et al*., 2013; Khadrawy *et al*., 2016; Khan *et al*., 2018). However, only the study Khadrawy *et al*., 2016 assessing this endpoint was taken forward for BMD analysis and not the other two because these were all single-dose studies/experiments. Other endpoints related to this period of exposure, namely ‘excitatory or inhibitory neurotransmitters (GABA, ASP, GLU, GLN, GLY and TAU)’

(Khadrawy *et al*., 2016), 'MAO activity' (Khan *et al*., 2018) and 'blood corticosterone production' (Fan *et al*., 2018) were not considered for BMD analysis due to a likelihood level of ALAN based on a Tier 2 study (Khadrawy *et al*., 2016) or considered of Inadequate evidence since only two single-dose Tier 1 or Tier 2 studies (Fan *et al*., 2018; Khan *et al*., 2018 and, respectively) were available.

109. The overall likelihood across all exposure periods, *i.e.* the highest likelihood given in the cluster Nervous system functionality, was Likely.

Behaviour

110. The cluster Behaviour comprises a number of different endpoints that relate to different types of actions or responses that can be observed in animals or humans. For the sake of clarity, the results of the WoE exercise will be presented by endpoint to demonstrate which behavioural measurements were susceptible to BPA at different exposure periods. The specific measurements that were included for the effects of BPA on behaviour belong to the endpoints of anxiety-related and emotional behaviour, learning and memory, locomotor activity and exploration, social behaviour and preference behaviour. Note that most studies cover more than one endpoint and some cover different stage of the animal life.

111. Overall, the cluster Behaviour comprises 17 studies performed in rats (of which two studies have indirect germline exposure through the male parent before conception, 10 studies relate to exposed animals during development until weaning, two studies relate to growth phase/young age and five studies relate to exposure in adulthood) and 13 studies performed in mice. Among these, two studies relate to indirect exposure of the male or the female parent before conception, seven studies relate to exposure during development until weaning, two to exposure in growth phase/young age and five to exposure in adulthood. In addition, there were three studies performed in monkeys and one study in prairie voles.

Developmental exposure (pre-natal and/or post-natal until weaning)

112. For effects of BPA in the developmental exposure period, 20 studies in total (in rats, mice, monkeys or prairie voles) were identified. From the rat studies, five were allocated to Tier 1 (Ferguson SA *et al*., 2014; Fujimoto *et al*. 2013; Fujimoto *et al*., 2015; Hicks *et al*., 2016; Johnson *et al*., 2016), three to Tier 2 (Hass *et al*., 2016; Wang C *et al*., 2016; Wang C *et al*., 2014), and two to Tier 3 (Rebuli *et al*., 2015; Sadowski *et al*., 2014a). One mouse study was allocated to Tier 1 (Luo *et al*., 2014), six to Tier 2 (Kumar and Thakur, 2017; Nagao *et al*., 2014; Naule *et al*., 2014; Picot *et al*., 2014; Sobolewski *et al*., 2014; Xin *et al*., 2018) and one to Tier 3 (Kundakovic *et al*., 2013). The study in cynomolgus monkeys (*Macaca fascicularis*) (Negishi *et al*., 2014) was in Tier 2 and the study in prairie voles (*Microtus ochrogaster*) (Sullivan *et al*., 2014) was allocated to Tier 3.

Anxiety/emotionality

113. For the endpoint anxiety/emotionality, five studies in rats, four studies in mice and one study in prairie voles were included. In most cases, the exposure started at implantation of the conceptus (2 studies in rats, 1 study in mice) or before or directly after mating of the parent animals (3 studies in mice, 1 study in rats). One study in rats examined animals exposed during the fetal period, whereas another study in rats and the study in prairie voles restricted exposure to the post-natal period.

114. No changes of anxiety or emotionality parameters were found in adult male or female rats in a Tier 2 study by Hass *et al*. (2016) with pre-natal and post-natal exposure covering a large dose range (25, 250, 5000, 50000 µg/kg bw per day). The test was performed in an EPM. The finding is supported by a Tier 3 study by Rebuli *et al*. (2015). These authors examined the behaviour of juvenile and adult rat offspring after pri-and post-natal exposure to 2.5, 25 and 250 µg/kg bw per day in the EPM and the Open Field and tested adult offspring also in the Zero Maze. None of the tests gave evidence for changes in anxiety-related behaviour following developmental BPA exposure. In contrast, Hicks *et al*. (2016) found evidence for increased anxiety in the Open Field in a single-dose study with exposure through the

drinking water throughout pregnancy and lactation (80– 195 µg/kg bw per day). Young adult male and female offspring from the BPA group spent less time in the centre area, away from the walls.

115. Fujimoto *et al*. (2013) did not find effects on anxiety either in the EPM in young adult rats of both sexes in a single-dose study (24 µg/kg per day) with exposure during the first pre-natal week. However, they observed an influence on depression-like behaviour in the Forced Swim Test in which males and females of the BPA group displayed a decrease in latency to immobility and males showed a prolonged duration of immobility.

116. Another indication for effects of developmental BPA exposure on anxiety-related parameters comes from a single-dose study (15 µg/kg bw per day) by Fujimoto *et al*. (2015). The authors reported an increase in the avoidance response to fox odour by adult males and females after exposure to BPA during the second half of the gestation period. Overall, the data in rats indicate effects of BPA only in the more stressful tests that examine the endpoint of anxiety/emotionality.

117. A single-dose Tier 2 study with mice detected increased anxiety in pre-natally and post-natally exposed males in the Open Field and the EPM at a dose of 50 µg/kg bw per day (Kumar and Thakur, 2017). The males were tested at the age of 8 weeks and the BPA group showed a lower inclination to visit the unprotected areas in both tests. Females were not examined in this study, but data from the single-dose study of Luo *et al*. (2014) conducted with a much higher dose (10000 µg/kg bw per day) indicate a comparable effect in female mice shortly after the end of exposure at weaning in the Open Field and in the EZM. The finding in females is supported by a Tier 3 study with pre-natal exposure. Females tested at the age of 8 weeks spent less time in the central area of the Open Field at the dose levels of 20 and 200 µg/kg bw per day (Kundakovic *et al*., 2013). However, male mice in this study exhibited a change in the opposite direction at all doses tested (2, 20 and 200 µg/kg bw per day). They spent more time in the unprotected area, a behaviour that resembles the normal pattern of control females.

118. Xin *et al*. (2018) did not observe increased anxiety in adult male mouse offspring from dams treated with 10 or 10000 µg/kg bw per day from before pregnancy until weaning when testing them in the EZM. However, they discovered an increase in depression-like behaviour (time spent immobile) in the Forced Swim Test which was similar in extent at both dose levels. Females did not show an increase in depression-like behaviour in this test.

119. Effects of BPA exposure appear Likely based on effects in Open Field, Elevated Maze and Forced Swim Test in Tier 1 and Tier 2 mouse studies and on findings in the Forced Swim Test and a predator odour avoidance test in rats.

Learning and memory

120. The endpoint learning and memory was examined in a single-dose study in mice (Tier 2) and five studies in rats with three or more dose groups and a control. Sobolewski *et al*. (2014) exposed pregnant/lactating mice from implantation until weaning to a dose of 50 µg/kg bw per day. Male and female offspring were tested as young adults in a novel object exploration and recognition test that examined response to novelty and short-term memory of familiar objects. In the BPA group both sexes showed a significant decrease in their initial exploration time spent with a novel object and the males also exhibited a decrease in overall exploration time. Females compensated the decreased duration of the exploration bouts with an increase in the number of approaches to the object. No effects were seen on short-term memory in this test. During fixed interval (60 s) reinforcement sessions, BPA-group males, but not females, exhibited significant reductions in response rates which could indicate an attention deficit or a lack of motivation to respond for food rewards.

121. In rats, Hass *et al*. (2016) did not identify learning deficits in a MWM in 4–6-month-old male or female offspring after developmental exposure (GD7 to PND22) to doses between 25 and 50000 µg/kg bw per day in their Tier 2 study.

122. In a Tier 3 study, Sadowski *et al*. (2014a) did not find changes in working or reference memory in BPA-exposed offspring tested as adults in a 17-arm radial

maze. The rats had been exposed from GD0 until PND9 to doses between 4, 40 or 400 µg/kg bw per day. However, with male rats tested after weaning and exposed only during pre-natal development (GD9 to GD20), Wang C *et al*. (2014) found an increase in 8-arm radial maze working memory errors compared with the control group. The effect was seen at all doses (50, 500, 5000 and 50000 µg/kg bw per day), especially during the first half of the 14-day trial period. A similar, albeit smaller, effect was seen for reference memory errors in this Tier 2 study. An increased error rate (incidence of sniffing incorrect holes) was also observed in a Tier 1 study by Johnson *et al*. (2016) in male and female rats exposed from GD6 to PND21 to a dose of 2500 µg/kg per day and tested as adults in a Barnes maze. In addition, the female rats exhibited an increased latency to locate the escape box in this test. No effects on Barnes maze performance were seen at lower doses (2.5 and 25 µg/kg per day).

123. In another Tier 2 study, a test for object recognition in weanling male rats exposed from GD9 to GD20 7612 resulted in a decrease of the object recognition index as a measure of short-term memory (1.5 hours) at 500, 5000 and 50000 µg/kg bw per day. Long-term memory (24 hours) was also impaired but only at 5000 and 50000 µg/kg bw per day. No effect on either measure was seen at the dose of 50 µg/kg bw per day (Wang C *et al*., 2016). This is compatible with the negative result in mice after developmental exposure to this low dose only (Sobolewski *et al*., 2014).

124. Based on findings of memory impairment, impaired novel object exploration/recognition and increased error rate an effect of BPA on these endpoints was judged to be Likely.

Locomotor activity/exploration

125. Regarding the endpoint locomotor activity/exploration the motor activity was studied either in the home cage of the animals or in an apparatus like the open field that allowed spontaneous movement and exploration in most cases.

126. Five studies in mice with either pre-natal (Nagao *et al*., 2014; Kundakovic *et al*., 2013) or pre-natal and post-natal exposure (Luo *et al*., 2014; Sobolewski *et al*., 2014; Xin *et al*., 2018) were available. Four of these studies did not identify an effect of BPA on this endpoint at doses between 2 and 10000 µg/kg bw per day. Only the Tier 3 study of Kundakovic *et al*. (2013) described a sexually dimorphic effect on distance travelled in the Open Field for males at all doses tested (2, 20 and 200 µg/kg bw per day) and for females at the two highest doses.

127. Similarly, out of eight studies in rats, only the study of Wang C *et al*. (2014), found a slight reduction between 10 and 30% in locomotor activity over a wide dose range (50, 500, 5000 or 50000 µg/kg bw per day) in males after pre-natal exposure. No clear dose dependency was observed. Females were not included in this Tier 2 study. In a different test setting, the same group (Wang C *et al*., 2016) detected no change in the time period the males spent exploring.

128. Other investigators did not report changes in motor activity of rats after either pre-natal (Fujimoto *et al*., 2015), pre-natal and post- natal (Rebuli *et al*., 2015; Hicks *et al*., 2016; Ferguson SA *et al*., 2014; Hass *et al*., 2016) or post-natal exposure (Fujimoto *et al*., 2013), although the study by Hass *et al*. covered a similar dose range (25, 250, 5000, 50000 µg/kg bw per day) as the study by Wang C *et al*. (2014).

129. The only other study in which an effect was observed was a Tier 3 study in prairie voles (Sullivan *et al*., 2014) that identified a reduction of activity after exposure during the second PNW to the dose of 50000 µg/kg bw per day in the female sex only. No effects occurred at lower doses or in males.

130. Overall, the available data suggest that an effect of BPA on this endpoint is Not Likely.

Social interaction

131. The effects of developmental exposure of BPA on various aspects of social behaviour were examined in rats (pre-natal and post-natal), mice (pre-natal and post-natal), prairie voles (post-natal) and cynomolgus monkeys (pre-natal).

132. No effects on sexual behaviour were seen in intact female rats exposed to doses of 2.5 or 25 µg/kg per day from implantation to weaning in a Tier 1 study (Ferguson SA *et al*., 2014) and in male mice exposed to doses of 50 or 5000 µg/kg bw per day from the fetal period until weaning in a Tier 2 study (Picot *et al*., 2014). BPA also did not change non-sexual social interactions in male and female rat offspring in a single-dose Tier 1 study with exposure through the drinking water amounting to 80–195 µg/kg bw per day (Hicks *et al*., 2016) or in a Tier 2 study with male mice exposed to 10 or 10000 µg/kg bw per day (Xin *et al*., 2018]). Only the Tier 3 study by Kundakovic *et al*. (2013) reported a decrease in chasing behaviour directed to cage mates in male mice at the highest dose tested (200 µg/kg bw per day).

133. A Tier 2 study in cynomolgus monkeys reported a decrease in social interaction in males after pre-natal exposure to BPA after subcutaneous exposure equivalent to an oral dose of about 1000 µg/kg bw per day (Negishi *et al*., 2014). In addition, the exposure to BPA abolished the known difference in discriminant scores that is present between control males and females.

134. In a Tier 3 study with a monogamous rodent species, the prairie vole, Sullivan *et al*. (2014) observed BPA effects on social interaction after exposure on PND8 to PND14. Females at a dose of 50000 µg/kg bw per day spent more time investigating a same sex stimulus animal, whereas males at 50 and 50000 µg/kg bw per day spent less time. Consequently, the normal sex difference of the response was lost or inverted. In the same study, female prairie voles seemed to show a decrease in formation of opposite sex partner preference bonding at all doses tested (5, 50 or 50000 µg/kg bw per day) based on a low number of females that spent time with a strange male in addition to their partner. This parameter was not affected in males.

135. There were no effects on social behaviour in Tier 1 and Tier 2 studies in rats and mice; weak effects were observed in a small single-dose level Tier 2 study in monkeys and in a Tier 3 study in female prairie voles. Overall, an effect of BPA on this endpoint was judged as Not Likely.

Preference behaviour

136. A Tier 1 (Ferguson SA *et al*., 2014) and a Tier 2 study (Hass *et al*., 2016) examined preference for sweet solution in male and female rats exposed during in utero development until weaning. No effect of BPA was detected in either study at doses equal to or lower than 5000 µg/kg bw per day. The dose of 50000 µg/kg bw per day used in the study of Hass *et al*. decreased the intake of saccharin solution by approximately 25% in females only, which may indicate masculinisation or de-feminisation of this behaviour at high doses.

137. BPA exposure to doses of 2.5 or 25 µg/kg bw per day did not change the preference for sodium chloride solution in males and females (Ferguson SA *et al*., 2014).

138. The available data indicate that changes in preference behaviour are Not Likely.

139. Overall, the CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA in the developmental exposure period (pre-natal and/or post-natal until weaning). Since the likelihood level for this cluster is Likely for the endpoints anxiety/emotionality (Xin *et al*., 2018) and learning and memory (Johnson *et al*., 2016; Wang C *et al*., 2016; Wang C *et al*., 2014), these were taken forward for BMD analysis and uncertainty. The endpoints anxiety/emotionality investigated in the studies by Kumar and Thakur (2017) and Luo *et al*. (2014) were not taken forward for BMD analysis because both are single-dose studies.

Developmental and adult exposure (pre-natal and post-natal in pups until adulthood)

140. No studies were available for this exposure period.

Growth phase/young age exposure

141. For this exposure period, two studies in rats (Bowman *et al*., 2015; Chen Z *et al*., 2018), two studies in mice (Zhou YX *et al*., 2017; Lou *et al*., 2013) and one study

in vervet monkeys (*Chlorocebus sabaeus*) (Elsworth *et al*., 2013) were identified. All studies were allocated to Tier 1.

Anxiety/emotionality

142. The endpoint anxiety/emotionality was studied in rats and mice. Chen Z *et al*. (2018) tested young adult rats that had been dosed orally with 40, 400 and 4000 µg/kg bw per day from weaning until PND49. They observed a statistically significant increase of latency to first entry into the centre area of an open field in males at 4000 µg/kg bw per day, as well as slight, non-significant increases in both lower dose groups. No effects were seen in females. Lou *et al*. (2013) examined adult male mice that had been given 10000 µg/kg bw per day from PND30 until PND70 in a dark/light test and the EPM and found evidence for increased anxiety at this dose with both tests.

143. Based on these findings, effects of BPA on anxiety/emotionality were judged as Likely for males.

Learning and memory

144. For the endpoint of learning and memory, studies in rats, mice and monkeys were available. In the study of Chen Z *et al*. (2018) male rats displayed reduced memory for platform location in the MWM in the mid and the high-dose group (400 and 4000 µg/kg bw per day). Memory was unaffected in females. In male mice treated with doses of 0.5, 50 and 5000 µg/kg bw per day from 4– 12 weeks of age, Zhou YX *et al*. (2017) found a decrease in learning performance in the high-dose group. These animals required an increased number of trials to qualify to the learning standard (90% correct response) in a Y maze test.

145. The other studies for this endpoint were conducted with subcutaneous exposure. In a study with rats employing only a single dose (40 µg/kg bw per day s.c., equivalent to 1428 µg/kg bw per day orally) Bowman *et al*. (2015) observed a decrease in overall exploration in males and in females, but no effect on spatial

memory performance. In addition, males but not females spent less time with novel objects than the control group. No effect was seen on the working memory of pre-pubertal male and female monkeys exposed to plasma levels of about 15 ng/ml (equivalent to 4500 µg/kg bw per day orally) for 30 days in the study by Elsworth *et al*. (2013).

146. The available data indicate a Likely effect of BPA on learning/memory for males but not for females.

Locomotor activity/exploration

147. No or no consistent effects on the other two endpoints examined for this exposure period, preference behaviour and locomotor behaviour, were found by Bowman *et al*. (2015) in their single-dose study with rats. Since this single-dose study was the only study available for these endpoints, there was Inadequate evidence to judge the likelihood of these effects.

148. Overall, the CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA in the exposure period Growth phase/young age. Since the likelihood level for this cluster is Likely for the endpoints anxiety/emotionality and learning and memory (in Chen Z *et al*., 2018 and Zhou YX *et al*., 2017, respectively) in males, these were taken forward for bench mark dose (BMD) analysis and uncertainty analysis.

Adult exposure (after puberty)

149. In this exposure group, 10 studies from three species were identified, four in rats, five in mice and one in vervet monkeys (*Chlorocebus sabaeus*). Three of the rat studies were allocated to Tier 1 (Fan *et al*., 2018; Fan *et al*., 2013; Nuñez *et al*., 2018), one to Tier 2 (Nojima *et al*., 2013). The database for mice consisted of one Tier 1 study (Xu XH *et al*., 2015), three Tier 2 studies (Picot *et al*., 2014; Khan *et al*., 2018; Xin *et al*., 2018) and one Tier 3 study with subcutaneous exposure (Liang *et al*., 2018) which was considered supportive for the endpoints anxiety/emotionality

and locomotor activity/exploration identified in Tier 1 studies. The single study in monkeys (Elsworth *et al*., 2015) was allocated to Tier 1.

Anxiety/emotionality

150. For the endpoint anxiety/emotionality, one study in rats and two studies in mice identified an increase of the level of anxiety in male animals. Fan *et al*. (2018) observed that male rats treated with the single dose of 50 µg/kg bw per day for 23 weeks spent about 40% less time in the centre area of an open field, whereas Xu XH *et al*. (2015) and Liang *et al*. (2018) found that male mice were not responsive in this type of test in their studies.

151. However, they displayed dose- dependent increases in anxiety or emotionality in two other tests, EPM and the FST. In the study by Xu XH *et al*. (2015), the effect was noted from the lowest dose tested (40 µg/kg bw per day) in the FST and for all doses in the EPM except the lowest one. Female mice showed either no or opposite effects (decreased anxiety/emotionality), consistent with possible sexual dimorphic effects of BPA in the brain.

152. An effect of BPA on the endpoint anxiety/emotionality was judged as Likely.

Learning and memory

153. With respect to learning and memory, the data available from single-dose studies in three species (rats, mice and vervet monkeys) indicate impaired cognition in males. Fan *et al*. (2013) observed that male rats given 50 µg/kg bw per day for 10 weeks in a small amount of food (5 g) exhibited increased swimming distance and escape latency during the learning phase for the location of the platform in the MWM test . In the probe trial that tested how well platform location had been memorised, they spent less time in the target quadrant and thus displayed a reduced capacity to remember. In the study of Khan *et al*. (2018), male mice showed a reduced attraction to novel objects after treatment with 10000 µg/kg bw per day for 5 weeks. This can be interpreted as a lack of discrimination between objects of different familiarity or, alternatively, as a fear of novelty. A study conducted with young adult male vervet

monkeys (Elsworth *et al*., 2015), pretrained in a two-choice spatial delayed response test, found a decrease in median per cent correct responses from 90% to about 83% 1 week after the start of exposure to 5555 µg/kg bw per day. The effect was reversible and full recovery was achieved 2 weeks after the end of the exposure.

154. Considering the above evidence, the effects of BPA on the endpoint learning/memory are judged as Likely.

Locomotor activity/exploration

155. No effects of BPA on locomotor activity in the open field test (OFT) was seen in a Tier 1 study in male rats with a single-dose level of 50 µg/kg bw per day (Fan *et al*., 2018), a Tier 3 study in male mice with oral equivalent dose levels of 8888, 88880, 888800 µg/kg bw per day (Liang *et al*., 2018), and a Tier 1 study in male and female mice with dose levels of 40, 400, 4000 and 40000 µg/kg bw per day (Xu XH *et al*., 2015). In the latter study, the animals were also tested in the EPM and a mirrored maze that examine entry into specific parts of the maze as an indicator for anxiety and may give some information on locomotor activity when total activity is considered. In both sexes, no effects were seen in the mirrored maze which is more similar to the OFT. BPA treatment also did not affect overall locomotion of female mice in the EPM, whereas males showed reduced overall activity at the two highest doses. However, it is considered that the lower activity of males resulted from the dose-related increase in anxiety and not from a direct impact on locomotor behaviour.

156. Nojima *et al*. (2013) conducted a single-dose study (Tier 2) with intraperitoneal exposure by minipump that measured spontaneous motor activity of male rats in their home cage. At a dose equivalent to 918 µg/kg bw per day orally they noted a difference in activity to the control group that was most pronounced shortly before and after the transition from dark to light phase. The deviation became statistically significant for the light phase only on days 11–12 after implantation of the minipump. However, the circadian activity pattern was not changed, and the BPA group showed slightly higher overall activity throughout the test period. As no pre-

treatment data are reported in the study it is not entirely clear if this is a substance effect or if the rats chosen for the BPA group already showed a higher home cage activity before the experiment started.

157. Overall, there is sufficient evidence to conclude that effects of BPA on the endpoint locomotor activity are Not Likely.

Sensory-motor coordination

158. Khan *et al*. (2018) found sensory-motor coordination deficits in male mice exposed to 10000 µg/kg bw per day. The animals showed a decreased performance on the rotarod and in a grip strength test. Since only one Tier 2 single-dose study was available the evidence was considered Inadequate.

Social interaction

159. Two Tier 2 studies in mice examined the effects of BPA on adult social behaviour. Xin *et al*. (2018) studied maternal behaviour in females on lactation day 1 after exposure from before mating and throughout pregnancy. They found no effects on nest building, pup retrieval or time spent in the nest at doses of 10 or 10000 µg/kg bw per day. In the study of Picot *et al*. (2014), sexual behaviour was affected in male mice exposed from week 8 to week 12 of age at a dose of 50 µg/kg bw per day but not at 5000 µg/kg bw per day. The males in the low dose required longer latencies to accomplish their first mount, intromission, thrust and ejaculation; in addition, the number of mounts with intromission and thrusts was also reduced. Therefore, it is judged as Likely that BPA affects male sexual behaviour.

Preference behaviour

160. Nuñez *et al*. (2018) examined the preference for sodium salt intake in male and ovariectomised (OVX) female rats during seven days of subcutaneous exposure to BPA in oral equivalent doses of 357, 1785, 3570 and 17850 µg/kg bw per day. The males decreased both their spontaneous water intake and the consumption of NaCl solution at all doses except in the lowest dose group and showed a decreased

preference for saline after fluid deprivation at 357, 3570 and 17850 µg/kg bw per day. No differences in preference were noted between the groups. For OVX females, decreased water intake was only observed at the highest dose, but they showed reductions in spontaneous salt intake and reduced preference for salt after fluid deprivation at the same doses as the males. The treated females showed a reduced preference for 2.7% NaCl at all doses when compared with the control group, but the effect was only statistically significant at 3570 µg/kg bw per day. The findings may indicate an effect of BPA on body fluid regulation that leads to differences in intake behaviour.

161. The BPA effects on this endpoint is judged as Likely.

162. Overall, the CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA exposure in adult males and females based on increased anxiety/depression-like behaviour in male mice (Xu XH *et al*., 2015 and Liang *et al*., 2018) and decreased anxiety in female mice (Xu XH *et al*., 2015); impaired male sexual behaviour in mice (Picot *et al*., 2014) and changes in salt preference in rats (Nuñez *et al*., 2018). Therefore, these endpoints were taken forward for BMD analysis and for uncertainty analysis .

163. The endpoints anxiety/depression-like behaviour in male rats (Fan *et al*., 2018) and impaired learning and/or memory in male rats (Fan *et al*., 2013), mice (Khan *et al*., 2018) and monkeys (Elsworth *et al*., 2015) were not taken forward for BMD analysis because the studies are single-dose studies.

Indirect (germline) exposure

164. Behavioural endpoints after exposure through the germline were examined in the offspring of rats and mice that had been treated with BPA before mating of the animals began. For effects through the female germline one study in mice (Xin *et al*., 2018) allocated to Tier 2 with a low and a high-dose group (10 and 10000 µg/kg bw per day) was available, in which the female parents had been exposed throughout their own development until weaning. This study did not identify any changes in the

two endpoints that were examined in their male offspring (anxiety/emotionality, locomotor activity) at any dose. Female offspring was not tested.

165. Two Tier 1 studies in rats (Fan *et al*., 2018; Fan *et al*., 2013) and one Tier 1 study in mice (Luo *et al*., 2017) dealt with possible consequences of the exposure of the male parent and compared only a single-dose group of BPA of either 50 µg/kg bw per day (in rats) or 10000 µg/kg bw per day (in mice) to a control group.

Anxiety/emotionality

166. The endpoint of anxiety/emotionality was examined by three different procedures in young adult rats offspring (Fan *et al*., 2018) and identified increased anxiety in females with all three tests (Forced Swim Test, Open Field and Elevated Maze). In the males, this finding was only obvious in the Forced Swim Test, which can be considered to impose a higher level of stress on the animals compared with the other testing procedures, open field and elevated maze. In the mouse study (Luo *et al*., 2017), these latter tests indicated increased anxiety in juvenile and young adult male offspring, respectively. Female mice were not tested.

167. Effects on this endpoint were judged as Likely.

Learning and memory

168. Spatial learning of male and female offspring was affected in the MWM in a rat study (Fan *et al*., 2013). The animals needed more time and longer swimming distances to locate the escape platform during the acquisition process. However, memory of the learned positions appeared affected only in females, not in males. As only a single-dose study was available, this evidence was considered Inadequate.

Locomotor activity/exploration

169. For the endpoint locomotor activity/exploration one study in rats (Fan *et al*., 2018) and one study in mice (Luo *et al*., 2017) were available. In young adult rats, no effects were observed on the distance travelled in the open field in either sex.

Juvenile male mice showed reduced overall locomotor activity in the same test. No data were obtained for female mice. In addition, a study with male mice (Xin *et al*., 2018) did not find any changes of exploratory/locomotor activity in a hole board test. An effect of BPA on locomotor behaviour was considered Not Likely.

Social interaction

170. Social interaction was studied in male mice only (Luo *et al*., 2017) and revealed a decrease in exploration time of same sex strangers and a reduced preference for social contact. As only a single- dose study was available, this evidence was considered Inadequate.

171. Overall, The CEP Panel assigned a likelihood level of Likely to the behavioural effects of BPA exposure through the male germline based on consistent findings in two species (rats and mice) for the endpoint anxiety/emotionality (in Fan *et al*., 2018 and Luo *et al*., 2017). However, both of these studies were conducted with only a single dose of BPA and none of them was taken forward for BMD analysis.

Overall cluster selection of the endpoints/studies for BMD analysis for Behaviour

172. Overall, the CEP Panel assigned a likelihood level of Likely to effects of BPA on behaviour in the exposure periods developmental until weaning, growth phase/young age, adult age and indirect (germline) exposure periods.

173. The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period developmental (pre-natal and/or post-natal development until weaning) for the endpoints anxiety/emotionality (Xin *et al*., 2018 and learning and memory (Johnson *et al*., 2016; Wang C *et al*., 2016; Wang C *et al*., 2014). Therefore, these endpoints were taken forward for BMD analysis.

174. The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period growth phase/young age for the endpoints anxiety/emotionality (Chen Z *et al*., 2018; Luo *et al*., 2013) and learning and memory (Chen Z *et al*., 2018; Zhou YX *et al*., 2017) in males.

175. The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period adult age for the endpoints anxiety/emotionality (Xu XH *et al*., 2015), sensory-motor coordination (Khan *et al*., 2018), and salt preference (Nuñez *et al*., 2018). Therefore, these endpoints were taken forward for BMD analysis.

176. The CEP Panel considered that the evidence from the studies available showed a Likely effect of BPA in the exposure period of the male germline for the endpoint anxiety/emotionality (Fan *et al*., 2018; Luo *et al*., 2017). These endpoints were not taken forward for BMD analysis because single-dose studies.

177. The overall likelihood across all exposure periods, *i.e.* the highest likelihood given in the cluster effects on behaviour, was Likely.

Integration of likelihoods from human and animal studies

178. Table 2 presents the overall likelihood per cluster for the human and animal stream separately, as well as the integration of the likelihoods from the human and animal studies for Neurotoxicity and developmental neurotoxicity.

Table 2: Integration of the human and animal studies for Neurotoxicity and developmental neurotoxicity

Human Stream	Animal stream	Integrated likelihood
Cluster: Neurodevelopment (behaviour after	**Cluster:** Behaviour	

developmental exposure)		
Exposure during Pregnancy Not Likely	Developmental (pre-natal and/or post-natal until weaning) Likely	
Exposure during Childhood Not Likely	Growth phase/young age Likely	
	Adult exposure (after puberty) Likely	
	Indirect (germline) exposure Likely	
Overall likelihood: Not Likely	Overall likelihood: Likely	Likely
Cluster: Neuromorphology	**Cluster: Neuromorphology**	
Not applicable	Developmental (pre-natal and/or post-natal until weaning) Likely	
	Growth phase/young age Likely	
	Adult exposure (after puberty) Inadequate evidence	
	Overall likelihood: Likely	Likely
Cluster: Nervous system functionality	**Cluster: Nervous system functionality**	

Not applicable	Developmental (pre-natal and/or post-natal until weaning) ALAN	
	Growth phase/young age Inadequate evidence	
	Adult exposure (after puberty) Likely	
	Indirect (germline) exposure Inadequate evidence	
	Overall likelihood: Likely	Likely

In vitro and Mechanistic studies

179. BPA effects are reported over a huge range of effective concentrations/doses (low nM and µg/kg per day to high µM and >1 mg/kg). This always needs to be considered; there may be qualitative as well as quantitative differences in mechanisms, depending on dose. In addition, apparent inconsistencies between studies may result from heterogeneity in study design (experimental models, route and window of exposure, dose of BPA, age at time of assessment, testing procedure, etc.).

180. It is clear that many of the reported effects of BPA on Neurotoxicity and developmental neurotoxicity endpoints are downstream pleiotropic effects (e.g. altered expression of many genes and proteins; ERK1/2 signalling, Wnt/β-catenin, Tmprss2, Foxa1, NGF, SOX2, Pax6, Grin2b, JNK, CREB and p53- mitochondrial apoptosis pathways, Gnrh1, Calbindin-D28, Kisspeptin (Kiss1), GNRH, DNA methylation (Dnmt, MECP2), hypotaurine, NMDA, GABA, oxytocin, serotonin and dopamine signalling, hypothalamic–pituitary axes (HPA, HPT and HPG), BDNF-NTRK2 neurotrophin system, etc.)

181. Details of upstream mechanisms, which are more likely to be BPA specific, are unclear, but receptor interactions are an obvious candidate mechanism. In particular, ER-dependent pathways have been implicated in a number of studies (Mhaouty-Kodja *et al*., 2018).

182. Mhaouty-Kodja *et al*. (2018) concluded that effects of BPA on learning and memory appear to be associated with the disruption of oestrogen-dependent pathways and of cerebral glutamate-NMDAR pathway (downstream targets leading to gene transcription of ERK, CREB, Brain-derived neurotrophic factor BDNF and synaptic proteins involved in synaptic plasticity).

183. It is unfortunately difficult to generalise data from studies reporting receptor-dependency of BPA effects, because steroid receptor properties and interactions are dynamic; substance effects can vary by tissue, life-stage and/or dose. In fact, much of the complexity of steroid receptor mechanisms has been revealed by research with BPA. Oxidative stress generation is an additional candidate mechanism for BPA effects on AChE activity and other endpoints. There is some evidence that BPA-induced oxidative stress is involved in brain functional effects at both low and high doses, but the data are not consistent.

184. In humans, Kondolot *et al*. (2016) sought to identify correlations between BPA levels and oxidant-antioxidant parameters in autistic and non-autistic children. No significant correlations were observed.

Neuromorphology

185. There was no human evidence available for the cluster Neuromorphology. Thus, the overall likelihood of effects of BPA for this cluster was scored Likely, based on the animal evidence.

186. Low-dose BPA-related reduction of dendritic spine density and/or cell number during development and/or growth phase (hippocampal CA1 and CA3) was judged as Likely.

187. BPA-induced mitochondria-related oxidative stress is one possible MoA.

188. Agarwal *et al*. (2016) concluded that pre-natal and post-pubertal low dose (40 µg/kg bw per day) of BPA impaired rat hippocampus mitochondrial fusion/fission dynamics and autophagy-mediated mitochondrial turnover, leading to increased oxidative stress, mitochondrial fragmentation, and apoptosis in hippocampal neural stem cells (NSCs), and inhibited hippocampal derived NSC proliferation and differentiation.

189. A possible mechanism for BPA effects on mitochondria is interaction with calcium channels.

190. Michaela *et al*. (2014) reported that nanomolar concentrations of BPA inhibited calcium current through T-type calcium channels (TCCs) in HEK cells. They suggested that this high-affinity low-efficacy inhibition may be caused by direct binding of BPA to TCCs in their resting state.

191. Chen *et al*. (2020) reported that TCC blockade (in C2C12 myoblasts) induced mitochondria-related apoptosis and reduced mitochondrial transmembrane potential (MMP), induced mito-ROS generation, and enhanced expression of mitochondrial apoptosis proteins.

192. Jiang *et al*. (2015) reported BPA-related decreased activities of mitochondrial respiratory complexes and abnormalities in mitochondrial morphology in rat cardiac myofibrils, including decreased mitochondrial volume density, reduced cristae density and increased vacuoles, after low-dose BPA (50 µg/kg bw per day) for 48 weeks. They considered it possible that the changes in mitochondrial function were a primary event to observed BPA-induced myocardial hypertrophy.

193. Mitochondria are the major source of cellular ROS, and there is evidence that neural stem cell activity is modulated by ROS (Hou *et al*., 2012; Adusumilli *et al*., 2021). BPA-induced ROS could be a possible MoA for the reported alterations in hippocampal cell numbers.

194. Overall, there is some evidence for a connection between BPA-induced mitochondria-related oxidative stress, possibly related to calcium channel blockade, and effects on neuromorphology.

Nervous system functionality

195. There was very limited human evidence available for the cluster Nervous system functionality, not fitting for the WoE. Thus, the overall likelihood of effects of BPA for this cluster was scored Likely, based on the animal evidence.

196. More specifically, Alavian-Ghavanini *et al*. (2018) investigated initially if low-dose developmental BPA exposure affects DNA methylation and expression of Grin2b (glutamate ionotropic receptor NMDA type subunit 2B) in brains of adult rats. They reported that developmental exposure to BPA changes the DNA methylation level in the Grin2b promoter, results in altered gene expression levels in female, but not in male, rats 1 year after the exposure had ceased. Extending their investigation to humans, the authors report that pre-natal BPA exposure was associated with increased methylation levels in girls. Attempting an indirect link they also report that low APGAR scores, a predictor for increased risk for neurodevelopmental diseases, were associated with higher Grin2b methylation levels in girls than boys.

197. Kundakovic *et al*. (2015) showed that pre-natal exposure to BPA induces lasting DNA methylation changes in the transcriptionally relevant region of the BDNF gene in the hippocampus and blood of BALB/c mice. Extending their work in humans, they examined BDNF IV DNA methylation in cord blood samples from the CCCEH cohort where high maternal BPA exposure had been associated with adverse behavioural effects in different sex groups. High pregnancy BPA levels (based on maternal spot urine) were associated with altered DNA methylation of two CpG sites in the human cord blood with an observed trend for a sex-specific effect of high BPA on CpG1A methylation and a significant sex-specific effect of high BPA exposure on CpG1B methylation levels.

198. Yang CW *et al*. (2014) identified candidate genes of neuronal development by implementing a gene ontology analysis and formed a reconstructed neuronal sub-

network. Subsequently, their gene expressions were determined in 20 umbilical cord blood samples dichotomised into high and low BPA level tiers. Two neuronal genes, sex determining region Y-box 2 (Sox2) and paired box 6 (Pax6), had preferentially downregulated expression in response to BPA exposure. Fetal cord blood samples had the obviously attenuated gene expression of Sox2 and Pax6 in high BPA group referred to low BPA group. Visualised gene network of Cytoscape analysis showed that Sox2 and Pax6 which were contributed to neural precursor cell proliferation and neuronal differentiation might be downregulated through sonic hedgehog (Shh), vascular endothelial growth factor A (VEGFA) and Notch signalling.

199. These results indicated that trans-placental BPA exposure downregulated gene expression of Sox2 and Pax6 potentially underlying the adverse effect on childhood neuronal development.

200. In animals, changes in neurotransmitters (GABA, noradrenaline NA, dopamine DA, 5-hydroxytryptamine 5HT serotonin) and OR were judged ALAN during developmental (pre-natal and/or post-natal until weaning) exposure.

201. At high doses (≥10000 µg/kg bw per day), BPA increased hippocampal neurotransmitters and also increased markers of oxidative stress and lipid peroxidation (Khadrawy *et al*., 2016). It is therefore possible that these neurotransmitter changes were downstream to (*i.e.* a result of) BPA- induced oxidative stress.

202. At low dose (10 µg/kg bw per day), mouse hippocampal DA was increased in males only, and NA was decreased in females only, without changes in GABA and GLN (Xin *et al*., 2018).

203. Low-dose BPA (≤2500 µg/kg bw per day) increased male OR density, and thus eliminated sex differences, in various sexually dimorphic brain nuclei (BNSTdl, VMH, paraventricular hypothalamic nucleus PVN (Witchey *et al*., 2019).

204. Given that these low-dose effects are gender-specific, it is possible that the MoA involves interactions with oestrogen (ER) and androgen (AR) receptors.

205. Altered AChE activity after adult exposure to BPA was judged Likely.

206. Low dose BPA (50 µg/kg bw per day) decreased AChE activity (−36%) in the rat hippocampus and impaired spatial memory (Fan *et al*., 2013). Given that BPA is reported by others to induce oxidative stress at low doses, this reduced AChE activity could be caused by oxidative stress, cf. Schallreuter *et al*. (2004).

207. At higher doses (20000–50000 µg/kg bw per day), BPA increased cortical AChE activity (by about 20–50%) (Khadrawy *et al*., 2016). Hippocampal AChE activity was similarly significantly increased after 10000 µg/kg for 6 weeks and 25000 µg/kg for 6 weeks, but not after 25000 µg/kg bw per day for 6 weeks, *i.e.* this was not a consistent effect.

208. A possible mechanism for the increased brain AChE activity at high doses is altered membrane fluidity leading to increased extracellular enzyme exposure, as proposed in the study by Macczak *et al*. (2017) for erythrocyte AChE activity ('AChE is located in erythrocyte membrane on phosphatidylinositol, and thus changes in membrane fluidity may lead to stronger exposure of this enzyme outside of the cell, which results in an increase of its activity.'). Such a mechanism implies cell damage, but brain histopathology, reported by others, was not measured in Macczak *et al*. (2017).

209. By contrast, Khan *et al*. (2018) reported reduced AChE activity (about −35%) in whole brain of adult mice dosed orally with BPA 10000 µg/kg bw per day for 30 days. Oxidative stress biomarkers in brain homogenates were also significantly increased (and neurobehavioural and cognitive performance was reduced). These study data support the hypothesis that oxidative stress is a causative upstream event leading to the observed decrease in AChE activity.

210. The reported relationship between both high-dose and low-dose BPA exposure and brain AChE activity is inconsistent, thus, it is not possible to propose a single mechanism, although there is some evidence suggesting that BPA-induced oxidative stress may be involved.

Behaviour

211. After the integration of the human and animal evidence, the overall likelihood of effects of BPA for the cluster Effects on behaviour was scored Likely.

212. Changes in behavioural endpoints are expressed downstream of functional and/or structural changes in the brain and are considered the apical indication that a living system has exhausted its innate ability to compensate for changes induced in the underlying processes.

213. Effects of BPA on learning and memory performances were judged as Likely in both sexes for two periods of exposure, *i.e.* the developmental (pre-natal and/or post-natal until weaning) and the adult period (after puberty), whereas it was limited to the males for BPA exposure in the growth phase and/or the young age.

214. Neurofunctional clusters interrelated with learning and memory performances were judged as Likely for the hippocampal and cortical AChE activity for an exposure occurring at the adult stage (after puberty), and ALAN for neurotransmitter systems in various parts of the brain following a developmental exposure (pre-natal and/or post-natal until weaning). Concomitant neuromorphological changes including dendritic morphology and spine density in hippocampus and cortex were also judged as Likely for the two earliest periods of exposure (development until weaning and growth phase and/or young age) and as ALAN for exposure limited to the adult stage (after weaning).

215. Effects of BPA on anxiety and depression-like behaviour were judged as Likely in all exposure periods: during pre-natal and/or post-natal exposure until weaning (in mice); during the growth phase and young age (in male rats and mice); in adult animals; and also, after indirect exposure through the germline of the male parent. Possible mechanisms include changes in corticosteroid regulation and in Wnt/β-catenin signalling.

Corticosterone regulation

216. Sex-specific functional alterations of the hypothalamic–pituitary–adrenal (HPA) axis and concomitant changes in anxiety-like behaviour have been described after developmental exposure to BPA. Chen F *et al*. (2014, 2015) reported a reduction in the HPA axis response to stress for females ('anti-anxiety-like' behaviour), whereas in males they found a hyperactivation. BPA-exposed males, but not females, had higher basal levels of serum corticosterone and adrenocorticotrophic hormone (ACTH), as well as an increase of corticotropin releasing hormone (CRH) mRNA in the hypothalamic PVN.

217. Pubertal female rats exposed to BPA exposure at 40 µg/kg bw per day during pregnancy and lactation showed increased basal corticosterone and reduced hypothalamic GR levels. A stress challenge elicited more anxiety-like behavioural coping and a weaker corticosterone response compared with control females (Panagiotidou *et al*., 2014).

218. In female rat offspring exposed to BPA at 40 µg/kg bw per day during pregnancy and lactation, Zhou *et al*. (2015) found a significant increase in both basal (morning) and peak (afternoon) corticosterone release compared with controls and increased basal and peak plasma ACTH at the same time points. In the hippocampus, mRNA expression for GR, mGlu2 and mGlu3 receptors as well as proteins levels of mGlu2/3 receptors were decreased, suggesting that both increased corticosterone levels and decreased signalling through hippocampal mGlu2/3 contribute to the anxiety and depression-like behaviour observed after BPA exposure.

219. The increased corticosterone production observed in BPA-exposed animals seems to involve impaired feedback from the hippocampus to the HPA axis which leads to increased levels in CRH and ACTH. The CRH signalling pathway was identified among the top scoring pathways in the transcriptome of the amygdala in male and female neonatal rats after pre-natal BPA exposure at 25 µg/kg bw per day (Arambula *et al*., 2018).

220. An activation of Cyp11A1 (P450scc) in the adrenal was shown by Medwid *et al*. (2016) in mice at 5000 µg/kg bw per day and by Lan *et al*. (2015) in mouse Y1

adrenal cortex cells in the BPA concentration range 50–1000 nM, and in rats injected subcutaneously with BPA (0.5 µg/kg bw per day for 3 days, equivalent to oral 17.85 µg/kg bw per day). In addition to this increase of adrenal steroid synthesis, an upregulation of corticosterone production could also occur in the neurons themselves; hippocampal neurons contain the complete pathway of corticosteroid synthesis and normally produce low levels of corticosterone (approximately 7 nM) that are sufficient to modulate synaptic plasticity (Hojo *et al*., 2011). Thus, a cell-autonomous overproduction could augment the adverse effects of circulating corticosteroids on synaptic plasticity and affective behaviours.

221. A possible mechanism for the effects of BPA on both corticosterone and anxiety is via ER-mediated altered hippocampal expression of FKBP5 (FKBP51), a GR binding protein that negatively regulates GR, which is upregulated by stress, and is connected to neuronal synaptic plasticity (Qiu *et al*., 2019). Increased Fkbp5 promoter methylation (decreased protein expression) has been reported to be associated with an anxiety phenotype and increased corticosterone levels in mice after pre-natal trauma (Plank *et al*., 2021). Kitraki *et al*. (2015) found an increase in DNA methylation of this gene at a BPA dose of 40 µg/kg bw per day in male rats, which probably resulted from a reduction of ERβ binding to a site in intron 5 of the gene.

222. Similar to BPA, increased corticosterone levels have been associated with a reduction in dendritic spine density in hippocampus and PFC and an increase in anxiety/depression-like behaviour (Wang G *et al*., 2013a).

223. In conclusion, it appears that the effects on the HPA axis at the molecular level, the morphological decrease of dendritic spine density in hippocampus and PFC, and the apical change in affective behaviour (anxiety/depression-like behaviour) are causally connected and delineate a MoA for BPA neurotoxicity. In the dose range between 2 and 40 µg/kg bw per day, BPA exposure increased serum corticosterone by 10–60%. It decreased dendritic spine density or dendritic spine synapses in layer II/III mPFC pyramidal cells and hippocampus for all exposure periods (lowest effective dose reported was 40 µg/kg bw per day) and increased

anxiety/depression-like behaviour (lowest effective dose reported at 10–20 µg/kg bw per day).

224. A MoA involving increased corticosterone production could be envisaged as follows:

• Binding of BPA to ERβ changes specificity for target genes (including Fkbp5 in hippocampus) imply a decreased Fkbp5 expression, which would lead to an increase in CRH and ACTH. This activates Cyp11A1 (P450scc) in mitochondria of adrenal cells (and possibly in hippocampal neurons), causing an increase of corticosterone production through the c-Jun JNK) signalling pathway, an increase of serum corticosterone and a corticosterone-dependent decrease in dendritic spine density associated with increased anxiety/depression.

225. Altered Wnt/β-catenin pathway activity is a further possible MoA. Arambula *et al*. (2018) identified Wnt/β-catenin signalling (four genes) as one of the top pathways affected by BPA in neonate amygdala at 25 µg/kg bw per day in males. BPA was shown to downregulate this pathway and to increase β-catenin degradation in rat brain and in cultured hippocampal neurons in studies by Liu ZH *et al*. (2014, 2015) and by Tiwari *et al*. (2015, 2016). BPA exposure upregulated GSK3β which phosphorylates β-catenin and marks it for degradation in the absence of ligand. As a consequence, nuclear translocation of β-catenin was decreased in the hippocampus and the subventricular zone (SVZ). Proliferation and neuronal differentiation of hippocampus-derived neural stem cell as well as hippocampal and subventricular zone neurogenesis were impaired (Tiwari *et al*., 2015). BPA-induced decreases in dendritic spine density were noted in the dentate gyrus and the CA1 area of the hippocampus by Liu ZH *et al*. (2015). This finding was replicated in cultured CA1 neurons with BPA concentrations of 10 nM or greater. Importantly, this *in vitro* effect was abolished by addition of a Wnt ligand (Wnt7a) to the culture, suggesting that Wnt is causally involved in the effect.

226. Wnt signalling together with BDNF cooperatively regulates dendritic spine formation; Wnt signalling inhibition in cultured cortical neurons disrupts dendritic

spine development (Hiester *et al*., 2013). BDNF is a direct target of Wnt signalling, being induced through Wnt-dependent TCF/LEF transcription factors (Yi *et al*., 2012) and then can exert a positive feedback on Wnt signalling through induction of Wnt2 which is sufficient to promote cortical dendrite growth and dendritic spine formation *in vitro*. BPA diminished the expression of LEF-1 and TCF mRNAs and proteins in the hippocampus of rats (Tiwari *et al*., 2015).

227. Activation of Wnt-dependent β-catenin signalling decreased the expression of steroidogenic genes (including Cyp11a1) in an adrenocortical cell line and caused a reduction in the release of corticosterone (Walczak *et al*., 2014). The inhibitory effect of BPA on the Wnt pathway may contribute to the upregulation of corticosterone production.

228. In addition to the canonical (β-catenin dependent) pathway, non-canonical Wnt pathways appear to be involved in BPA effects on neurons. Liu ZH *et al*. (2014) reported a dose-dependent decrease of the canonical ligand Wnt7a while the non-canonical ligand Wnt5a increased in hippocampal dentate gyrus homogenates. Wnt5a has been shown to modulate mitochondrial dynamics in cultured hippocampal neurons and to promote fission of mitochondria through recruitment of the fission regulator Drp1 from the cytosol to the mitochondria. The change in mitochondrial morphology was associated with significant increases in cytosolic and mitochondrial Ca2+ levels (Godoy *et al*., 2014).

229. Agarwal *et al*. (2016) showed that BPA increased mitochondrial fragmentation in the hippocampus (including dentate gyrus and CA regions) of the rat brain at a dose level of 40 µg/kg bw per day and in hippocampal NSC-derived neuron cultures at a concentration of 100 µM.

230. In conclusion, these studies support the involvement of canonical and non-canonical Wnt pathways in the effects of BPA on functional and structural parameters in the brain and their downstream consequences for behavioural endpoints.

Conclusion on hazard identification for Neurotoxicity and developmental neurotoxicity of BPA

231. In the EFSA opinion 2015 (EFSA CEF Panel, 2015), a likelihood level of ALAN was assigned to neurological, neurodevelopmental and neuroendocrine effects of BPA in a WoE approach. This was based on the results from prospective epidemiological studies examining children exposed to BPA during the pre-natal period. The studies provided evidence for an association with sex-dependent behavioural problems but not sufficient proof for a causal link, due to inconsistent findings across studies. Animal studies, while indicating a possible impairment of brain functions and behavioural parameters such as anxiety-like behaviour, learning and memory, social behaviour and sensory-motor function, presented methodological shortcomings as well as inconsistent results from different studies.

232. Therefore, the CEP Panel decided not to take these effects forward to derive the toxicological RP but used them in the analysis of uncertainty for hazard characterisation and risk characterisation.

233. In the current assessment, epidemiological evidence derived from newly available longitudinal studies examining children with exposure during pregnancy or post-natally did not suggest any endpoints related to neurodevelopment as critical for risk assessment. The children were followed for various time periods, up to the age of 10 years. Although some statistically significant associations were observed, none of them occurred in more than one study and subsequent research failed to replicate the results.

234. The available cross-sectional studies produced some evidence for associations of BPA exposure and various neurodevelopmental endpoints in children but are not considered robust enough on their own to support an adverse association.

235. With respect to the animal studies, the results of the present evaluation extend the previous database and indicate possible effects of BPA during development and in adults mainly on anxiety and depression- related behaviours,

learning and memory, as well as on dendritic spine density and AChE activity in the hippocampus and the PFC. Loss of spines in these brain regions has also been observed in association with impaired cognitive ability and mood disturbances in a number of other animal models. Thus, it can be assumed that the reduction of dendritic spine density forms the structural basis for the behavioural changes induced by BPA. The mechanisms that underly the effect on spine density are less clear.

236. Spine formation may be affected by various signals, including neurosteroids, neurotransmitters and their receptors, synaptic plasticity-promoting proteins, signalling pathways and oxidative stress, most of which have also been identified in studies that examined possible MoAs for BPA. This includes for example reductions in the expression of ERs, overproduction of corticosterone, downregulation of NMDA receptors, changes in PSD-95 expression and interference with Wnt/β-catenin signalling.

237. Overall, the CEP Panel considers that the different effects observed on brain structure, neurochemistry and functional outcome, *e.g.* behaviour, can be integrated into a convincing picture that indicates the existence of a neurotoxic hazard of BPA in developing, growing and adult animals.

238. Using a WoE approach, the CEP Panel assigned a likelihood level of Likely to the effect of BPA on anxiety/emotionally, learning and memory, salt preference, dendritic spine density and AChE activity. Therefore, these endpoints were brought forward for BMD analysis.

Conclusions

239. The newly available literature data indicate that the central nervous system is a target of toxicity for BPA.

240. Within the HOC Neurotoxicity and developmental neurotoxicity, the evaluation of the human data considered endpoints from the cluster neurodevelopment. In the

animal studies, three clusters of endpoints were identified: neuromorphology, nervous system functionality and behaviour.

241. Based on the human data, it was concluded that the evidence for an association between BPA exposure and impaired neurodevelopment was Not Likely.

242. Based on the animal data, all three neurotoxicity clusters showed effects that were judged as Likely:

- In the neuromorphology cluster, Likely effects were found for the endpoints dendritic spine density of pyramidal cells in hippocampus (CA1 and dentate gyrus areas) after developmental exposure and for the endpoints number of neurons in hippocampus (CA1 and CA3 areas), and dendritic spine density in pyramidal cells in the medial part of the PFC after exposure during the growth phase/young age.

- In the nervous system functionality cluster, a Likely effect on the endpoint AChE activity during the adult exposure period was identified.

- In the behaviour cluster, Likely effects were noted for the endpoint anxiety/emotionality during all exposure periods (developmental, growth phase/young age, adult and exposure through the male germline). Furthermore, the endpoint learning/memory showed a Likely influence of BPA from developmental and growth phase/young age exposure, and effects on sensory-motor coordination and salt preference were considered Likely in adults.

243. The mechanisms of action that link the identified effects of BPA on various endpoints of brain structure, function and development have not been sufficiently explored in the literature to draw conclusions. There is evidence for an involvement of steroid-hormone-dependent pathways (oestrogen, androgens, corticosterone); oxidative stress, mitochondrial function and calcium regulation; gene expression

changes through DNA methylation and other signalling pathways (canonical and non-canonical Wnt pathways, kinases).

Questions for the Committee

Do Members have any comments on:

a) The human data

b) The animal data

c) The WoE and integration of this endpoint.

d) The overall conclusions reached.

e) Any other comments

Secretariat

February 2022

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Abbreviations

AChE	Acetylcholinesterase
ACTH	adrenocorticotrophic hormone
ADHD	attention deficit hyperactivity disorder
ALAN	as likely as not
ASP	aspartate
AVPV	anteroventral periventricular nucleus
BSID-II	Bayley Scales of Infant Development-II
BMD	Benchmark dose
BPA	bisphenol A
BNSTp	bed nucleus of stria terminalis
BDNF	Brain-derived neurotrophic factor
C	Clusters
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
cPen	caudal periventricular nucleus
CRH	corticotropin releasing hormone
DA	dopamine
DLT	dark light test
DLPFC	dorsolateral prefrontal cortex
EPM	elevated plus maze
Exp	Exposure periods
EPM	elevated plus maze
FST	forced swimming test

GABA	Gamma amino butyric acid
GLN	Glutamine
GLU	Glutamate
GLY	Glycine
HOC	health outcome category
HPA	hypothalamic–pituitary–adrenal
MAO	Monoamino-oxidase
MMP	mitochondrial transmembrane potential
MWM	Morris water maze
mPFC	medial prefrontal cortex
NA	Noradrenaline
NSCs	neural stem cells
OFT	open field test
OTR	Oxytocin receptor
OVX	ovariectomised
PFC	Prefrontal cortex
PDD-NOS	Pervasive Developmental Disorder-Not Otherwise Specified
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonic acid
POMC	Pro-opiomelanocortin
PNW	post-natal week
PVN	paraventricular nucleus
RAM	radial arm maze
rPen	rostral periventricular area

RCMAS	Revised Children's Manifest Anxiety Scale
Shh	sonic hedgehog
SVZ	subventricular zone
TAU	Taurine
TCCs	T-type calcium channels
VEGFA	vascular endothelial growth factor A
VMH	ventromedial hypothalamus
VM	ventral mesencephalon
VMWM	Virtual Morris Water Maze