Bisphenol A: Studies by Stump et al. (2010) and Ryan et al. (2010) provide no indications for adverse effects on neurological development and behaviour

Bisphenol A: Studien von Stump et al. (2010) und Ryan et al. (2010) ergeben keine Hinweise für nachteilige Auswirkungen auf die neurologische Entwicklung und das Verhalten

BfR Opinion Nr. 035/2010, 29 July 2010

Stellungnahme Nr. 035/2010 des BfR vom 29. Juli 2010

The assessment of potential health risks possibly arising from the hormone-like effects of the industrial chemical bisphenol A has repeatedly been subject to controversial scientific debate over the past years. This led amongst others to the review of various risk assessments from European agencies (EFSA, 2006; EU, 2008) as regards any needs for adjustments. During the recently updated risk assessment of bisphenol A (EU, 2008) according to Council Regulation (EEC) No. 793/93, the Member States did not reach consensus on how concerns for potential developmental neurotoxicity of bisphenol A could be adequately considered. A re-evaluation of 4 critical studies performed by the Norwegian Food Safety Authority (VMK, 2008) came to the conclusion that the respective studies do not provide sufficient evidence for setting a robust lower NOAEL for the risk assessment of bisphenol A. The Authority recommended to carry out a GLP compliant study according to OECD test guideline 426 in order to eliminate any uncertainty regarding potential developmental effects of BPA at low doses. The experimental study performed by Stump et al. was published in the spring of 2010. The Federal Institute for Risk Assessment (BfR) has evaluated the results of the study as well as results of the study by Ryan et al. (2010) which complements the study by Stump et al.

Stump et al. conducted their study on bisphenol A according to the required regulatory standard test design on rats. The results obtained with these testing conditions did not provide any indications of adverse effects on neurological and behavioural development in the offspring. The study comprised also testing of very low dosages. Dietary administration of bisphenol A did not reveal indications for so-called “low-dose effects”.

The test design of the study of Ryan et al. had a particular focus on the investigation of estrogen-sensitive endpoints, a pivotal issue in the current scientific debate. The results revealed no adverse effects in the low-dose range on behaviour and the development of female rat offspring whose dams were treated with bisphenol A during gestation and lactation. In contrast, female offspring from dams treated under the same conditions with ethinyl estradiol showed irreversible abnormal behaviour, impaired fertility and malformations of the external genitalia.

According to BfR, the results of the two studies do not substantiate the concerns for a specific toxic potential of bisphenol A adverse to neurological and behavioural development.

Die Bewertung möglicher Gesundheitsrisiken durch hormonähnliche Wirkungen der Industriechemikalie Bisphenol A ist während der letzten Jahre mehrfach Gegenstand kontroverser wissenschaftlicher Diskussionen gewesen. Diese gaben u.a. auch Anlass, Risikobewertungen europäischer Behörden (EFSA, 2006, 2008, EU, 2008) auf notwendige Anpassungen zu überprüfen, wie zuletzt in dem aktualisierten Risikobewertungsbericht (RAR) nach dem EU-Altstoffverfahren (EWG Nr. 793/93) (EU, 2008) geschehen. In diesem erzielten die Mitgliedsstaaten keinen Konsens, wie vorhandene Verdachtsmomente auf ein entwicklungsneurotoxisches Potenzial bei der Sicherheitsbewertung von Bisphenol A berücksichtigt werden sollten.

Eine erneute Evaluation durch die Norwegische Food Safety Agency (VMK, 2008) führte zu dem Ergebnis, dass die Verdachtsstudien nicht genügend aussagekräftig waren. Sie gab die Empfehlung, für Bisphenol A eine Studie durchzuführen, die dem internationalen Standard der OECD Prüfrichtlinie 426 zur Entwicklungsneurotoxizität und den Bestimmungen für die Gute Labor Praxis entspricht, um die Unsicherheiten über mögliche neurologische Entwicklungs- und Verhaltenseffekte aufzuklären. Diese experimentelle Studie von Stump et al. wurde im Frühjahr 2010 veröffentlicht. Das Bundesinstitut für Risikobewertung (BfR) hat die Ergebnisse sowie eine Studie von Ryan et al. (2010), die die Untersuchungen von Stump et al. ergänzt, bewertet.

Stump et al. führten die Untersuchung zu Bisphenol A mit dem geforderten regulatorischen Standardtest an Ratten durch. Die Ergebnisse erbrachten unter den verwendeten Testbedingungen keine Anzeichen für schädigende Auswirkungen auf die Gehirn- und Verhaltensentwicklung der Nachkommen. Die Studie schloss auch die Prüfung der Wirkung von sehr geringen Dosierungen ein. Die mit dem Futter täglich verabreichten Bisphenol-A-Gaben erbrachten keinen Anhalt für Effekte im sogenannten "Niedrig-Dosis-Bereich".

Die Untersuchung von Ryan et al. legte mit ihrem Studiendesign besonderes Augenmerk auf die im Fokus des wissenschaftlichen Meinungsstreits stehenden östrogen-sensitiven Endpunkte. Die Ergebnisse zeigten im Niedrig-Dosis-Bereich keine nachteiligen Auswirkungen auf das Verhalten und die Entwicklung der weiblichen Nachkommen von Ratten, deren Mütter während der Trächtigkeit und Laktation mit Bisphenol A behandelt worden waren. Im Gegensatz dazu wiesen die weiblichen Nachkommen der unter gleichen Bedingungen mit Ethinylestradiol behandelten Positivkontrolle irreversible Verhaltensstörungen, eine beeinträchtigte Reproduktionsfunktion und Fehlbildungen der äußeren Geschlechtsorgane auf.

Aus Sicht des BfR lässt sich aus den beiden neuen Studien kein Verdacht auf ein spezifisches schädigendes Potenzial von Bisphenol A für Verhalten und neurologische Entwicklung ableiten.

1 Introduction

The toxicological properties of bisphenol A (BPA) have been a matter of scientific debate and controversy for many years now. For more than 10 years BPA has been evaluated in several hazard and risk assessment procedures in Europe and worldwide.

The compound is known to have weak estrogenic properties[1] and therefore is considered to represent a so-called '*Endocrine Disruptor'*. It is claimed by some parties that very low doses of BPA induce adverse health effects (so-called '*low-dose effects'*), and it is controversially debated, whether this issue would be of relevance for the human health hazard and risk assessment of BPA. Up to now, there is no common appreciation on the term low-dose, respectively low-dose effects. Both are used similarly and simultaneously.

Low-dose or "low-dose effects" might be understood as

[1] Bisphenol A binds to estrogen receptors ERα and ERβ. In comparison to 17β-estradiol the binding affinity of bisphenol A is 10,000-fold lower for both ER subtypes and the estrogenic activity in various *in vitro* tests, such as e.g. proliferation assays, gene-reporter assays and prolactin release assays, is generally 3-5 orders of magnitude lower. Also, in *in vivo* screening tests for estrogenic activity, such as the uterotrophic assay, estrogenicity of bisphenol A was much weaker when compared to ethinyl estradiol (Gould et al. (1998), Kuiper et al. (1998), EU (2003), Kanno et al (2003))

(i) low doses/low (internal) body burdens achieved as a consequence of exposure to low environmental contamination,

(ii) *experimental* dosages, which are well below those, that are normally tested in regulatory studies, respectively dosages below a cut-off dose of 5 mg/kg bw/d (Melnick et al., 2002), respectively dosages below the reference dose (RfD) of the US EPA (50 µg/kg bw/day), which typically means dosages in the lower µg-range, or

(iii) *any kind of effect* that has been detected after administration or exposure to any kind of low doses of BPA.

While in the past the debate concentrated on questionable effects observed on the prostate in young male mice offspring after intrauterine exposure to BPA, resulting from treatment of dams during gestation with BPA in the low µg-dose range, nowadays the debate focuses on possible effects on behaviour in mice and rat offspring and morphological changes in their brains after exposure to BPA in the low µg-dose range.

2 Evaluation of bisphenol A in the European regulatory framework

In order to derive acceptable intake levels within the context of food safety, a first evaluation of BPA was conducted by the Scientific Committee on Food (SCF) in 2002, and based upon the data base available at that time a *temporary* tolerable daily intake (TDI) of 0.01 mg/kg bw/d had been suggested. A three-generation reproductive toxicity study in rats had been identified as the key study (Tyl et al., 2002), providing a NOAEL of 5 mg/kg bw/d (based on body weight effects) as a point of departure for risk assessment. Further, in 2006 the EFSA (AFC Panel[2]) performed a comprehensive risk assessment, and a TDI of 0.05 mg/kg bw/d was established (EFSA, 2006). At that time, a two-generation reproductive toxicity study in mice had been identified as the key study (Tyl et al., 2008a), also providing a NOAEL of 5 mg/kg bw/d based on liver effects (increased incidence of centrilobular hepatocyte hypertrophy of minimal to mild severity in adult F0 and F1 males and F1 females). Within the available database at that time 9 studies containing data on the investigation of neurobehavioural development (Table A) had been considered for evaluation.

With special attention to age-dependent differences in the toxicokinetics of BPA in humans as compared to rodents and with special regard on human fetal as well as human neonatal exposure situations, the EFSA risk assessment of BPA had been updated again in July 2008 and the former TDI (0.05 mg/kg bw/d) had been re-confirmed (EFSA, 2008).

In addition to the European food safety regulations, BPA had been evaluated within the regulatory framework of chemicals safety according to Council regulation (EEC) No. 793/93 on the evaluation and control of existing chemicals. For BPA as a candidate of the 3rd Priority List according to Commission Regulation (EC) No. 143/97, a first risk assessment report with UK being the rapporteur has been published in 2003 (EU, 2003). In April 2008, this report had been updated (EU, 2008), because a further piece of information for the hazard assessment of BPA – a two-generation reproductive toxicity study in mice with exposure to both, low dose ([µg/kg bw/day] as well as high dose [mg/kg bw/day] BPA had become available (Tyl et al., 2008a). This study had been performed on the request of the EU Member States, since the human health assessment part from 2003 had concluded that further research was needed to resolve the uncertainties concerning the potential for BPA to produce adverse effects on development at low doses.

[2] Panel on Additives, Flavourings, Processing Aids and Materials in Contact with Food

3 Background to the new studies on neurodevelopmental toxicity of bisphenol A

In the updated risk assessment report of the European Union (EU, 2008) besides the new data from the two-generation reproductive toxicity study in mice (Tyl et al., 2008a) any newly available toxicological information for the various endpoints had been included, which also comprised information on the endpoint developmental neurotoxicity. A total of 34 studies were identified containing some information on developmental neurotoxicity, none of them had been performed according to common testing standards. They had been included in the update (EU, 2008) and had been considered for the re-evaluation of the toxicological properties of BPA (Table A). The final weight of evidence assessment of these studies, however, concluded that confidence in the reliability on the developmental neurotoxicity data is low because of limitations in the design and reporting in all available studies. The assessment of the consistency across the available studies concluded that there is no discernable and reproducible pattern of the behavioural testing results. Finally it was stated that no conclusions can be drawn from these studies.

Denmark, Sweden and Norway, however, did not agree with this conclusion [footnote on page 120, EU (2008)] claiming that four studies [Negishi et al. (2004), Carr et al. (2003), Ryan and Vandenberg (2006), Adriani et al. (2003)] were sufficiently reliable for regulatory use. In their caveat, they express the opinion that the effects observed in the four studies would indicate a possible risk for developmental neurotoxicity of BPA at very low exposure levels (0.1-0.25 mg/kg bw/d) and therefore advocated to either use these limited data as point of departure for quantitative risk assessment or finalise the risk assessment with conclusion (i) There is need for further information.

At the same time when EFSA and the European Union updated their risk assessments, evaluations on BPA became also available from the United States and from Canada, such as from Health Canada/Bureau of Chemical Safety (2008), the US National Toxicology Program (NTP, 2008), the US Food and Drug Administration (US FDA, 2008) and the Californian EPA (CalEPA, 2009). These evaluations also gave special attention to studies providing indications for a toxic potential on adverse effects on neurodevelopment and behaviour. Whereas some evaluations have a critical view on the validity and significance of the respective studies leading to the opinion that without any confirmation of the findings by well-designed experimental studies, data from these studies would not be appropriate to serve for regulatory decisions, others consider at least some studies as relevant for the assessment of BPA concerning neurodevelopmental and behavioural toxicity.

Different to other risk assessments, the US National Toxicology Program, which does not represent a regulatory agency, does not consider approaches to *quantitatively* assess risks but rather uses a *qualitative,* weight of evidence based system consisting of a categorical five-level scale to express their conclusions and characterise the likelihood of an adverse human health effect resulting from exposure to a substance (the qualifiers are: serious concern>concern>some concern>negligible concern for adverse effects). Based on several of the studies reporting effects on neurodevelopment and behaviour from BPA exposure at low doses, the NTP finally came to the conclusion (NTP, 2008) that there is ”some concern” for adverse effects due to developmental toxicity for fetuses, infants and children (effects on brain, behaviour and prostate gland)[3]. Health Canada/Bureau of Chemical Safety also con-

[3] Note: the NTP Expert Panel Evaluation of Soy Infant Formula containing estrogenic isoflavones, in late 2009 came to the conclusion that there is *minimal* concern for adverse developmental effects in infants fed soy infant formula

sidered some of the rodent studies with BPA as relevant for the assessment of neurodevelopmental and behavioural effects. Subsequent to their review of these studies, however, they questioned the potential clinical and toxicological significance and/or relevance of the findings for human health risk assessment and recognised that there is need for further research in this area (Health Canada/Bureau of Chemical Safety (2008a).

An overview of references that had been considered in various evaluations is presented in Table A. The results of some of these studies have been interpreted as giving concern for hormonally induced disorders of development and behaviour after exposure to low doses (µg-range) of BPA, which would mainly affect sexual differentiation of the nervous system and sexually dimorphic behaviour.

Due to the reservations expressed by three Nordic EU Member States, the Scientific Committee for Food Safety on request of the Norwegian Food Safety Agency in June 2008 published an in-depth evaluation of the four studies that had been indicated as relevant for the EU risk assessment by Denmark, Sweden and Norway (VKM, 2008). The panel came to the conclusion that the four studies do not provide sufficient evidence for setting a robust lower NOAEL for BPA than the current EFSA NOAEL of 5 mg/kg bw/day that was derived from the 2-generation reproductive toxicity study in mice (Tyl et al (2008a). It was recommended to conduct a GLP compliant study according to OECD test guideline 426 using a broad concentration range from the very low doses up to those with known maternal effects in order to eliminate any uncertainty regarding potential developmental effects of BPA at low doses.

Two recent studies on neurodevelopmental and behavioural toxicity of BPA are now available: an oral (dietary) GLP study according to OECD test guideline 426 (Developmental Neurotoxicity Study) in Sprague Dawley rats which was commissioned by the American Chemistry Council (Stump et al., 2010), and a non-GLP peri-/postnatal gavage study in Long Evans rats with a design similar to that of ICH guideline 4.1.2. The latter was performed at the National Health and Environmental Effects Research Laboratory (NHEERL) of the US EPA (Ryan et al., 2010). Additional information concerning effects of low doses of BPA on development and behaviour from an earlier GLP study according to OECD test guideline 416 (Two-generation reproduction toxicity study) on Sprague Dawley rats, which had been extended to investigate respective endpoints, is also considered for this evaluation (Ema et al., 2001).

4 Discussion of the Stump et al. (2010) and the Ryan et al. (2010) study

In the following section, the Stump et al. (2010) and the Ryan et al. (2010) study will be discussed in detail:

4.1 Stump et al. (2010) Developmental Neurotoxicity Study of Dietary Bisphenol A in Sprague-Dawley Rats

The test was performed as a dietary study using female Sprague Dawley rats. Mated females were exposed continuously via diet from gestation day 0 (GD 0) until weaning on postnatal day 21 (PND 21). With this test-setting the offspring is exposed indirectly (pre- as well as postnatally [via lactation]) and in part directly via participation in food consumption. Effects on brain function and morphology were investigated using five dietary concentration levels (0, 0.15, 1.5, 75, 750 and 2250 ppm). Based on food consumption data, the feed concentrations corresponded to mean uptakes of BPA of the dams of 0, 0.01, 0.12, 5.85, 56.4 and 164 mg BPA/kg bw/d during gestation, and to 0, 0.03, 0.25, 13.1, 129 and 410 mg BPA/kg bw/d during lactation. The five dietary concentrations were selected in order to cover

low dose exposure (intakes in the lower µg-range) for which neurodevelopmental effects had been reported in other studies. High doses were selected to detect systemically toxic effects. The highest dose tested (2250 ppm) was selected on the basis of a previously performed range finding study (Stump, 2009).

The study consisted of two cohorts, each composed of 12 females/dose group (Table B).

All females were allowed to litter and rear their offspring until weaning on PND 21, when dams were sacrificed. After culling of the litters on PND 4 the offspring was assigned to various sub-cohorts (A, B, C) for the evaluation of the following endpoints:

- Detailed clinical observations (DOC) at various time points in an open field (PND 4, 11, 21, 35, 45 und 60)
- Motor activity at various time points (PND 13, 17, 21 and 61)
- Auditory startle at PND 20 and at PND 60
- Learning and memory at PND 22 and at PND 62
- Brain weight, neuropathological assessment and morphometry at PND 21 and at PND 72

Besides, the surface righting response as a measure of reflex ontogeny was determined for all pups beginning with PND 2. In the offspring assigned to sub-cohort A, endpoints for sexual maturation (balanopreputial separation, vaginal patency) were determined.

Motor activity testing was performed automatically using a so-called Kinder Scientific Motor Monitor System (personal computer-controlled cage system counting the interruptions of a series of infrared photo beams as activity counts). At the different time points of the longitudinal observation the same individuals was tested at any point in time.

Auditory startle response was also tested automatically using a so-called Kinder Scientific Startle Monitor System. With this setting any increased or decreased responsiveness to an acoustic stimulus is tested. At the two different time points of the longitudinal observation the same individuals were tested at any point in time.

Learning and memory abilities were tested using a water-filled eight-unit t-maze, so-called Biel-maze. Within this setting animals have to traverse the maze by swimming and escape by locating a submerged platform. The time required to traverse the maze is determined and the number of errors for all trials to escape are counted. Altogether two different ways (path A and path B) to escape the maze were offered and tested, and sequential and reverse offer of these possibilities also allowed testing of *sequential* learning and memory.

For the neuropathological examinations the animals were perfused after sacrifice in situ with a fixative and the whole brains removed, and weight, length and width of the brains were taken. From the animal group sacrificed on PND 72 also parts of the peripheral nervous system were taken for histopathological examinations. Sections from all major brain regions (including olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons and medulla oblongata) were submitted to histopathological evaluation. Morphometric analysis of various brain regions, including at least two measurements from the neocortical, hippocampal and cerebellar areas, were performed automatically using an image-capturing computer system.

In addition to these endpoints, data on clinical observations, body weight/body weight gain and food consumption, which are standard data also for other regulatory tests, were collected.

4.1.1 Results

In dams the exposure up to and including the highest dietary concentrations did not affect maternal survival, clinical observations, pregnancy rate, gestation period and littering, maternal body weight and food consumption during lactation. However, at the highest dietary concentration (2250 ppm) an initial reduction in food intake (GD 0-7) was observed as well as a 22.4 % lower ($p<0.001$) body weight gain (GD 0-20) in comparison to the control group. Also after exposure to diet containing 750 ppm BPA, a reduction in body weight gain (GD 0-20) of 9.5 % ($p<0.02$) was observed. Maternal liver and kidney organ weight as well as histopathological investigations of organs did not reveal any differences compared to the control group.

In the offspring no effects on the number of pups born, live litter size, sex ratio, body weight at birth or postnatal viability were observed. A reduction in postnatal body weight gain (PND 7-14) was revealed for the 2250 and 750 ppm groups. Lower body weights were determined in the 2250 ppm group during PND 11-21 (8.3 % lower body weight ($p<0.001$) compared to controls) and in the 750 ppm group during PND 14-17 (5.6 % lower body weight ($p<0.05$) compared to controls). Development of surface righting reflex and the mean age at which surface righting reflex was attained was similar across all groups (exposed and non-exposed). Further, follow-up of growth and development after weaning did not reveal any differences between groups in terms of body weight gain and time of attainment of sexual maturation.

No test-substance related effects were noted during detailed clinical observations of the offspring with one exception: On PND 11 two out of 44 animals from the 750 ppm group and four out of 46 animals from the 2250 ppm group exhibited irregular movements of limbs, head and/or body which were recorded as *convulsions* and as *pop corn seizures*. Similar irregularities were not observed in any other animals nor were they observed in the six indicated animals on any other date. These six animals did also not show any other abnormalities during the detailed clinical observations nor did they show any irregularities in the tests for motor activity, auditory startle or learning and behaviour. Comparison to laboratory historical control data showed that the indicated findings (convulsions and pop corn seizures) were also observed in untreated control, although at a lower incidence than in the actual test. Due to these findings, a further small scale study applying the highest dietary concentration was additionally performed to establish the reproducibility of this finding. For this follow-up study a total of 27 litters were used and the detailed clinical observations were performed on twice the animal number (2 pups/sex/litter) that had been used for the main study. Substance-related effects observed on the body weight of dams and of offspring were similar as observed in the main study. None of the pups, however, exhibited any of the findings observed before in the main study (convulsions and pop corn seizures), and detailed clinical observations did not reveal any other effects. As the findings from the main study had not been reproduced in the follow-up study, the behavioural abnormalities as observed in six animals on PND 11 in the study were not considered to be substance related.

For the test on *motor activity* historical control data as well as data on the test performance and validity – as normally required by the test guideline – were provided with the test report. Haloperidol served as a positive control for reducing motor activity and nicotine and amphetamine served as a positive control for increasing motor activity. In the BPA exposed offspring, no differences in the pattern of activity counts (determined at 6 intervals of 10 minutes each and for a total 60 minutes) at the various dates could be observed in comparison to the

unexposed controls. Further, no effect on the pattern of habituation could be observed when animals were tested subsequently and at various ages.

Also for the test on *auditory startle response* historical control data as well as data on the test performance and validity - as normally required by the test guideline - were provided with the test report. Data obtained with chlorpromazine served as a positive control for an impairment of the response and data obtained with nicotine, amphetamine or 8-OH-DPAT (8-hydroxy-*N*,*N*-dipropyl-2-aminotetralin), an experimental 5-HT receptor agonist, served as a positive control for an improvement of the response. In the BPA exposed offspring, no differences in the response amplitude at any age (PND 20 or PND 60) or in the pattern of habituation over the test sessions at either age could be observed in comparison to the controls.

Also for the tests on *learning and memory* historical control data as well as data on the test performance and validity – as normally required by the test guideline – were provided with the test report. Data obtained with scopolamine served as a positive control for prolonging latency and increasing erroneous trials in the Biel maze. The swimming ability on the first day of testing was similar across all groups (BPA exposed and non-exposed) for either age. Also, the number of errors in escaping the maze via either path as well as after reverse sequence did not reveal significant differences across groups at either age tested.

In the offspring sacrificed at weaning (PND 21 or at a later stage (PND 72)) no differences were found in mean length, width or in organ weight of the brain. The examination of serial sections did not reveal any histopathological or morphometric changes.

In summary it can be concluded, that in this dietary study on Sprague Dawley rats substance related effects were induced in the dams as well as in their offspring in terms of reduced body weight and reduced body weight gain after exposure to concentrations of 750 and 2250 mg BPA/kg diet. This is plausible, since from the preceding dose range finding study it was reported that exposure to dietary concentrations of 7500 ppm BPA led to the termination of this dose group ahead of schedule due to clear cut maternal toxicity in terms of maternal body weight loss, and that exposure at the lower exposure group (2500 ppm) also led to maternal weight loss and/or reduced maternal weight gain and in the offspring to lower body weight at birth, reduced weight gain and reduced postnatal survival. Dietary concentrations of $\leq$ 75 ppm BPA (according to a daily intake of approximately 5.85 mg/kg bw/day during gestation and of approximately 13.1 mg/kg bw/day during lactation), however, did not affect any of the endpoints.

Except the effects observed on body weight gain at the two higher dietary concentrations no further effects had been observed in this test. The testing of standard endpoints able to detect effects on the brain and/or neurological and behavioural development, however, did not reveal any impairment at very low exposure levels and no impairments were seen at exposure levels that had been associated with effects on body weight. Thus the results of this study do not provide evidence for neurodevelopmental toxicity of BPA at the exposure levels that had been tested.

4.1.2 BfR comment

The study of Stump et al. (2010) meets the requirements of the neurodevelopmental toxicity study as requested by the Norwegian Food Safety Authority consequently to their re-evaluation of the four crucial non-regulatory studies that had been considered as valid and relevant for quantitative risk assessment by three Nordic EU countries during the EU risk assessment of BPA. The test followed a guideline-conform standard protocol using oral ad-

ministration via diet (an application route of human relevance) and addressed relevant endpoints for the detection of a specific toxic potential harmful to brain and/or neurobehavioural development. With this protocol (OECD 426, adopted in 2007), which is the counterpart of the US EPA guideline OPPTS 870.6300, used in the US and in Canada for the approval of pesticide active substances, more than 130 test reports and a comprehensive retrospective evaluation are available (Makris et al., 2009, Fitzpatrick et al., 2008, Crofton et al., 2008, Raffaele et al., 2008; Holson et al., 2008, Tyl et al., 2008b). Thus, this test protocol has already been applied routinely and broad experience has been gained with it.

As has been exemplified for other regulatory testing on BPA (e.g. Tyl et al. 2002, 2008a), also in this current developmental neurotoxicity study supplementary investigations would have been reasonable. For instance, such additional investigations could have aimed at measuring systemically available BPA after the dietary exposure by sampling blood and determining BPA plasma concentrations in exposed animals. Such information could have been helpful e.g. in verifying the systemic bioavailability of BPA after dietary administration and in providing quantitative information on the internal exposure from external administration. It has been complained repeatedly, that the guideline studies with dietary administration of BPA cannot be easily compared to other studies on BPA using e.g. oral administration via gavage or via drinking water. Moreover, data on internal exposure (e.g. based on plasma levels) would allow for a better comparison of study results.

However, the determination of test substance plasma levels is not yet an integral (optional) part of the OECD test guideline 426 nor is it recommended in the standard protocol. Likewise, also for many of the so-called “academic” studies (non GLP and/or non guideline-conform studies) in which effects had been observed in the low dose range after oral administration (often with gavage or administration using a pipette) no data on internal exposure levels are available. The latter also applies to the studies of Negishi et al. (2004), Carr et al. (2003), Ryan and Vandenberg (2006) and Adriani et al. (2003), the studies considered as relevant for quantitative risk assessment in the EU risk assessment report from April 2008 (EU, 2008) by Denmark, Sweden and Norway.

4.2 Ryan et al. (2010) In Utero and Lactational Exposure to Bisphenol A, in Contrast to Ethinyl Estradiol, Does Not Alter Sexually Dimorphic Behavior, Puberty, Fertility and Anatomy of Female LE Rats

This investigation was performed as an oral (gavage) study with female Long Evans (LE) rats. Pregnant dams were treated daily during gestation starting on GD 7 and lactation until PND 18 with corn oil (vehicle control) and with either BPA or with ethinyl estradiol (EE) as a positive-control estrogen at various doses. With this test-setting the offspring is exposed indirectly for both the period of sexual differentiation of the organs of the reproductive system as well as during the neonatal period of sexual differentiation of the brain. Besides a vehicle control group, three groups of animals were treated with BPA at 2, 20, or 200 µg/kg bw/d and additional animal groups treated as positive controls with EE (0.05, 0.15, 0.5, 1.5, 5, 15 and 50 µg /kg bw/d).

The study consisted of two cohorts. The first block used 169 animals with 13-29 pregnant females per treatment group. The second block comprised 82 animals with 6-14 pregnant females per group (Table C).

The authors give the following rationale for the study design and selection of BPA doses:
(1) determination whether low doses of EE or of BPA applied during pregnancy and lactation would alter the morphology of the organs of the reproductive system
(2) addressing the concerns that had been expressed by the National Toxicology Program (NTP, 2008) related to adverse effects on brain development and behaviour (i.e., to determine whether maternal exposure to very low doses of BPA would affect any for rodents well characterised sexually dimorphic behaviour, sexual maturation and reproductive functions in females). Sweet preference and lordosis behaviour, well characterised behaviours and specific for the rat species, which depend on neonatal hormonal (estrogenic) priming, were used as specific behavioural tests for hormonally-related sex specific differences.

A first set of data from this study upon effects of this treatment schedule on the reproductive morphology of the male offspring (effects had been observed in the EE exposed males, whereas no effects had been observed in male offspring of the BPA treated females) had been presented in a preceding publication (Howdeshell et al. 2008).

The following endpoints were investigated:

After weaning (PND 22 – PND 25) the dams were sacrificed and the numbers of implantation sites determined. On PND 2 and PND 14 in males and in females pup body weight, sex and anogenital distance were determined. On PND 14 also the numbers of retained areolae/nipples were determined (in the male offspring due to their comparatively higher androgen levels the nipple anlagen normally regress already in utero). After weaning of the female offspring (PND 23), development of body weight gain and attainment of sexual maturation (vaginal opening) were followed.

For the investigation of effects on reproductive function a sub-cohort of male and female offspring was built from the second block after weaning. These animals were mated for a period of at least four months and studied for the production of litters. If at least one litter was produced during this period, the mated animals were considered to be fertile. Also, the total number of pups born during this period was determined (fecundity).

Investigations on the effects upon sex-specific behaviour:

1. Test on sweet preference

For this test, the test animal was offered two bottles with different fluids as their normal source of drinking water. One bottle contained pure water and the other contained a saccharine solution. In a preceding pilot study on adult male and female LE rats, the expected sex-specific difference (females prefer sweetened drinking water) had been tested and confirmed. The sweet preference in the main test was calculated as the amount of saccharin solution (in females about 80 %, in males about 40 %) in relation to the total amount of fluid intake. During the preceding pilot study the animals had been offered saccharin solutions of either 0.25 % or of 0.5 % as an alternative to pure water, whereas in the main study the animals had been tested only for 0.25 % saccharin solution as an alternative to pure water in order to calculate sweet preference.

2. Test on sex-related differences in spontaneous activity in a so-called figure-8 maze

According to unpublished observations female LE rats in the respective test-setting figure-8 maze) exhibit a higher 24 hour activity level than their male counterparts. With this test-setting animals are allowed to explore the maze for a period of 10 hours while dark and activity levels (interruptions of photo beams) are counted automatically on an hourly basis.

In a preceding first pilot study on adult male and female LE rats, the expected sex-specific difference (females are more active than males) was tested and verified.
In a second pilot study the impact of depleting endogenous estrogens from circulation by ovariectomy was tested, in order to determine any changes in the activity patterns of females in direction to that of males. Following ovariectomy and determination of resulting activity patterns, these females were assigned to three groups and treated for 14 days with either corn oil (vehicle control) or 175 or 275 µg EE/kg bw/d for artificial replacement of endogenous estrogen levels. The activity levels after the various replacement schedules were determined again, and based on the results of this pilot investigation the exposed females from the main study were treated for 14 days with 275 µg EE/kg bw/d – a schedule which allowed re-constitution of the typical activity pattern in untreated ovariectomised females - and the activity patterns of the estrogen-substituted females was determined again.

3. Test on lordosis behaviour

Background for this test: according to Gorski (1986) female rats that have been defeminised by neonatal estrogen exposure display low levels of lordosis behaviour as adults. The test on lordosis behaviour was performed on ovariectomised females and followed a protocol with which females were treated orally with 50 µg EE/kg bw on the 1st and 2nd day and with 0.5 mg progesterone/kg bw before noon on the 3rd day with the test on lordosis behaviour starting in the afternoon (dark phase) as soon as females were placed with a male. The lordosis behaviour was recorded for a period of 2 minutes and calculated as a so-called lordosis quotient LQ determined as number of lordosis/number of mounts.

In a preceding pilot study adult untreated ovariectomised LE rats had been administered EE orally at various doses with this protocol to determine an optimal oral dose that induces lordosis behaviour reliably (LQ=1) in control females.

Furthermore, reproductive organ morphology was assessed in the female offspring after sacrifice at the age of 10 months, when external genitalia were measured and evaluated for abnormalities.

4.2.1 Results

After treatment of dams with EE, clear-cut effects were observed on maternal body weight gain during gestation. Beginning with EE doses of ≥ 1.5 µg/kg bw/d, a statistically significantly reduced weight gain was determined when compared to the dams of the control group. Further to this, with EE doses of 50 µg/kg bw/d also a statistically significant reduction in uterine implantation sites could be determined. In the dams that had been treated with BPA, no effects on body weight gain or on the numbers of uterine implantation sites were observed.

In the offspring of dams that had been treated with 15 or 50 µg EE/kg bw/d, effects were seen on numbers of newborns/litter (reduced) and on offspring viability (increased fetal and neonatal mortality). In addition to this, in the high dose group (50 µg) also reduced pup body weight (in males as well as in females) and in female pups reduced anogenital distance was detected. Furthermore, in the female offspring reduced body weight gain persisted at least up to PND 23. In the offspring of dams that had been treated with BPA, no such effects were observed.

In the offspring of groups that had been treated with EE, an acceleration of sexual maturation was observed. For the groups administered doses of ≥ 5 µg/kg bw/d, premature vaginal patency occurred which was in addition attained at lower body weight when compared to controls. In the offspring of groups that had been treated with BPA, no effects on sexual maturation were observed. The fertility of the female offspring and their total numbers of offspring were significantly reduced in the groups of dams that had been treated with EE doses of ≥ 5 µg/kg bw/d.

Testing for sweet preference revealed that female offspring from the dams that had been treated with 5 and with 50 µg EE/kg bw/d had a significant change in saccharin preference corresponding to that of males. No change in saccharin preference was observed in female offspring of dams that had been exposed to BPA.

During the tests for sex-related differences in spontaneous activity, in the 1st pilot study, untreated adult males exhibited a statistically significantly ($p<0.01$) lower activity level than female animals, as expected. In the 2nd pilot study, the ovariectomised and subsequently EE substituted females presented activity levels comparable to those of intact females (demonstrating that for adult females the *actual* estrogen is the determining factor for this behaviour). Prenatal exposure, however, to either EE or to BPA did not affect the spontaneous activity levels in both ovariectomised females and in ovariectomised estrogen substituted females.

The test on lordosis behaviour performed on ovariectomised, estrogen stimulated (50 µg EE/kg bw/d) females revealed that female offspring from dams that had been treated with 15 or 50 µg EE/kg bw/d did no longer respond to the stimulation, i.e., lordosis behaviour could not be re-activated, indicating that prenatal estrogen exposure had led to irreversible reorganisation/defeminisation of the brain. In contrast to this, no effects on lordosis behaviour were detected in female offspring of BPA treated dams.

The examination of the reproductive organs revealed that 63-100 % of the female offspring from dams, which had been treated with 5-50 µg EE/kg bw/d, exhibited abnormalities of the

external genitalia (urethral slit, vaginal tunnel, cleft phallus). In the offspring derived from the highest dose group (50 µg), these abnormalities were so severe that it was not possible to determine time of sexual maturation (vaginal patency). In female offspring derived from the BPA treated dams, no such abnormalities were observed.

In summary it can be noticed that in this study in utero and lactational exposure to ethinyl estradiol induced permanent changes in behaviour, reproductive function and morphology of reproductive organs of female Long Evan rats. Treatment of pregnant dams with doses of 5-15 µg EE/kg bw/d produced the following findings in their female offspring: abnormalities of the external genitalia, premature vaginal patency and permanent changes in behaviour that is specific to female rats such as lordosis behaviour and sweet preference in direction of de-feminisation. Further, fertility and the capacity to produce viable offspring were also reduced in these F1 females. For the overall study, logistic regression curves had been calculated for the individual endpoints, which were affected in the F1 female (and male) offspring of the treated dams. Besides indications that the F1 females are more seriously affected than their male siblings, it was shown that the impairment of female fecundity and malformations of the female genitalia were affected most sensitively (ED_{50}=2.6 µg EE/kg bw/d). In contrast to this, the pre-/postnatal treatment of pregnant rats with 2, 20, or 200 µg BPA/kg bw/d did not adversely affect any of the endpoints that had been investigated (in dams as well as in their female offspring). Thus the results of this study do not provide evidence that BPA in the dose range of 2-200 µg/kg bw/d induces changes in the LE rat because of its estrogenic properties, respectively in terms of an estrogenic action on estrogen-sensitive endpoints or in terms of significance for the organisational role of estrogens in the development of sexual behaviour in the LE rat. Further, the results did not provide evidence that BPA in the dose range of 2-200 µg/kg bw/d induces adverse effects on the female reproductive system.

4.2.2 BfR comment

The investigation of Ryan et al. (2010) is considered as a very valuable supplementation to the information obtained from the standard developmental toxicity study on BPA performed by Stump et al (2010), since it is suspected that prenatal exposure to this compound may potentially induce irreversible behavioural changes due to its estrogenic properties. Substances with endocrine active properties are generally under suspicion for potential impact on sexual differentiation of the brain. Up to now, however, the investigation of possible effects on sex-specific differences in behaviour is not an element of current standard test protocols. In addition to this, no generally accepted methodological repertoire has been compiled and approved for these investigations. For the Extended One-Generation Reproduction Toxicity Study, a new test guideline which is currently under development at the OECD and which in future may be considered one of the key standard test protocols for the identification of so-called endocrine disrupters, a certain sub-cohort of animals is foreseen in order to specifically investigate developmental neurotoxicity (DNT). The implementation of endpoints for the investigation of sex-specific differences in behaviour within this DNT cohort is currently being discussed. Emanating from the BPA case, Li et al. (2008) have recently developed some guidance on methodological aspects of test-settings designed to investigate hormonally mediated effects on brain development including sexual differentiation of the brain. In their opinion, the most important issues to be addressed are: the route of administration (oral route as the most relevant for humans), the litter as the experimental unit (not the individual pup) and within-test verification that the applied animal model is sensitive to the chemical under investigation, as indicated e.g. by concurrent positive-control chemicals. At length, all these aspects were considered and realised in the Ryan et al. (2010) study, which finally did not provide evidence for adverse effects of BPA in LE rats following perinatal exposure to low doses (2-200 µg) of BPA.

In general, there are difficulties in interpreting the behavioural data obtained from rodents concerning their possible relevance to humans (Wilson and Davies, 2007; McCarthy, 2008). For instance, some types of sexually dimorphic behaviour observed in *male* rodents are considered estrogen-dependent types of behaviour that are organised during the early phase of sexual differentiation of the brain, whereas other types of sexually dimorphic behaviour – like rough and tumble play – are considered to be androgen dependent. It is generally assumed, that in rodents the so-called estrogen signalling pathway is of higher significance for the sexual differentiation of the brain than the androgen signalling pathway, whereas in humans and non-human primates the androgen signalling pathway is predominant in sexual differentiation of the nervous system (Li et al. 2008).

Furthermore, when examining possible toxicological hazards emanating from bisphenol A or other endocrine active compounds, concentrating preferentially on the evaluation of *estrogenic* effects is unlikely to be sufficient. There are clear indications, in particular from studies at the molecular level (le Maire et al., 2010), that such substances may interact with a variety of other nuclear hormone receptors (NHR), which are not specifically involved in reproductive processes or in steroid biosynthesis and metabolism, as for instance the estrogen receptors (ERs) and the androgen receptor (AR) are. Interactions demonstrated for NHRs, which are involved in the control of multiple signalling pathways such as retinoid X receptors (RXR), or with NHRs that are related to regulating energy metabolism such as peroxisome proliferator-activated receptors (PPARs) and also estrogen related receptors (EERs), point to the need that future hazard assessments of such chemicals should also evaluate possible adverse effects beyond the pattern of estrogenic effects.

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Table A – Compilation of studies reporting effects of bisphenol A on brain and behaviour as considered or specifically highlighted in various evaluations

	Studies evaluated by **EFSA** (2006)	Studies evaluated by **EU** (2008) X1): studies considered sufficiently reliable for regulatory use/quantitative risk assessment by Denmark, Sweden and Norway	Studies reviewed in detail by **US FDA** (2008)	Studies classified as having "high utility" by the CERHR expert panel on bisphenol A **NTP** (2008)	Studies specifically addressed in the Screening Assessment for Bisphenol A /Characterisation of risk to Human Health section by Environment Canada **Health Canada** (2008b)	Studies reviewed in detail by Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch **Health Canada** (2008a)
References						
Aloisi et al.(2002)		X				
Adriani et al. (2003)	X	X1)	X		X	
Carr et al. (2003)		X1)	X		X	
Ceccarelli et al. (2007)		X	X			X
Della Seta et al. (2005)					X	
Della Seta et al. (2006)		X	X	X		
Dessi-Fulgheri et al. (2002)	X	X				
Ema et al. (2001)		X				
Facciolo et al. (2002)		X				
Facciolo et al. (2005)		X				
Farabollini et al. (1999)		X				
Farabollini et al. (2002)	X	X				
Fujimoto et al. (2006)		X				
Funabashi et al. (2004)		X				X
Gioisa et al. (2007)			X		X	
Honma et al. (2006)		X				
Kawai et al. (2003)	X	X				
Kawai et al. (2007)		X				

	Studies evaluated by **EFSA** (2006)	Studies evaluated by **EU** (2008) X1): studies considered sufficiently reliable for regulatory use/quantitative risk assessment by Denmark, Sweden and Norway	Studies reviewed in detail by **US FDA** (2008)	Studies classified as having "high utility" by the CERHR expert panel on bisphenol A **NTP** (2008)	Studies specifically addressed in the Screening Assessment for Bisphenol A /Characterisation of risk to Human Health section by Environment Canada **Health Canada** (2008b)	Studies reviewed in detail by Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch **Health Canada** (2008a)
References						
Kubo et al. (2001)		**X**				
Kubo et al. (2003)	**X**	**X**				
Kwon et al. (2000)		**X**		**X**		
Laviola et al. (2005)		**X**	**X**			**X**
Nakamura et al. (2006)		**X**				
Narita et al. (2006)		**X**				
Narita et al. (2007)		**X**				
Negishi et al. (2003)		**X**				
Negishi et al. (2004)	**X**	**X**1)	**X**	**X**	**X**	**X**
Nishizawa et al.(2005a)						**X**
Nishizawa et al.(2005b)						**X**
Nishizawa et al. (2003)						**X**
Mizuo et al. (2004a)	**X**	**X**				
Mizuo et al. (2004b)		**X**				
Palanza et al. (2002)	**X**	**X**	**X**	**X**	**X**	
Patisaul et al. (2006)		**X**				
Patisaul et al. (2007)		**X**				
Porrini et al. (2005)		**X**				
Rubin et al. (2006)					**X**	
Ryan et al. (2006)		**X**1)	**X**	**X**	**X**	**X**
Suzuki et al.(2003)	**X**					
Tando et al. (2007)		**X**				

Table B: Overview of essential components of the study of Stump 2009

<table>
<tr><td>Title of study</td><td>A dietary developmental neurotoxicity study of Bisphenol A in rats
20 September 2009, WIL Research Laboratories, Study No WIL-1860056</td></tr>
<tr><td>Authors</td><td>D. G. Stump</td></tr>
<tr><td>Method</td><td>OECD TG 426, US EPA OPPTS Guideline 870.6300</td></tr>
<tr><td>Species</td><td>Sprague Dawley rat</td></tr>
<tr><td>Treatment period</td><td>mated females exposed from gestational day 0 (GD) through lactation to postnatal day 21</td></tr>
<tr><td>Route of exposure</td><td>oral, diet

offspring potentially exposed to BPA in utero, as well as via lactation as well as directly via food during the latter portion of the lactation period</td></tr>
<tr><td>Dosing</td><td>bisphenol A (BPA)

<table>
<tr><td>BPA in diet (ppm)</td><td>0</td><td>0.15</td><td>1.5</td><td>75</td><td>750</td><td>2250</td></tr>
<tr><td>mean intake during gestation (mg BPA/kg/d)</td><td>0</td><td>0.01</td><td>0.12</td><td>5.85</td><td>56.4</td><td>164</td></tr>
<tr><td>mean intake during lactation (mg BPA/kg/d)</td><td>0</td><td>0.03</td><td>0.25</td><td>13.1</td><td>129</td><td>410</td></tr>
<tr><td>Number of dams</td><td>24/23</td><td>24/23</td><td>24/24</td><td>24/24</td><td>24/24</td><td>24/24</td></tr>
</table>

study consisted of two cohorts with 12 females per group each starting approximately 4 weeks apart

exposure levels selected to range from low doses used in some published studies reporting developmental neurotoxicity to a high dose that was anticipated to result in systemic toxicity in the pregnant rat (high dose selection based on previous range-finding study), 5 dietary concentrations tested

all females allowed to litter and raise offspring until PND21
litters culled (to 4 pups/sex/litter) on PND 4, following culling pups selected and assigned to
Subset A: 1 pup/sex/litter
Subset B: 1 pup/sex/litter
Subset C: 1 pup/sex/litter</td></tr>
<tr><td colspan="2">Endpoints evaluated</td></tr>
<tr><td>Maternal observations</td><td>clinical observations
food consumption</td></tr>
</table>

	body weight detailed clinical observations (DOC) on GD 10, 15 on PND 10, 21 gross necropsy on PND 21 with macroscopic examination as well as numbers of implantations and organ weights of *liver* & *kidney*
Offspring observations	all pups: sex, clinical observations, body weight surface righting response beginning on PND 2
Subset A	detailed clinical observations (DOC) on PND 4, 11, 21, 35, 45, and 60 evaluation for developmental landmarks: for balanopreputial separation: beginning on PND 35, for vaginal patency: beginning on PND 25 **auditory startle response** on PND 20 and 60 (longitudinal assessment) **motor activity** on PND 13,17, 21, and 61 **learning and memory** (Biel maze) beginning on **PND 62** necropsy on PND 72 and in situ perfusion: brain weight from all subset A pups brain morphometric and neuropathological evaluations from 10 pups/sex/group
Subset B	**learning and memory** (Biel maze) beginning on **PND 22** gross necropsy after completion of learning and memory evaluations
Subset C	necropsy on PND 21 and in situ perfusion: brain weight from all subset C pups brain morphometric and neuropathological evaluations on 10 pups/sex/group
Subset D	(all non-selected F1 offspring) gross necropsy on PND 21
Results	
Maternal and pregnancy data	pregnancy rates: 95.8%,95,8%, 100%, 100%, 100%, 100% in controls, 0.15, 1.5, 75, 750, 2250 ppm groups maternal survival unaffected, no BPA-related changes in clinical observation

	2250 ppm: ↓ maternal body weight gain (22.4%) during gestation (GD 0-20) 750 ppm: ↓ maternal body weight gain (9.5 %) during gestation (GD 0-20) effects on body weight gain associated with reduced mean food intake during lactation: no effect on body weight gain and food consumption no effects on gestation length or parturition no effects on reproductive performance of the dams at necropsy on PND 21: no effects on liver and kidney of the dams
Neonatal and offspring data	no effects on number of pups born, live litter size, sex ratio, or on postnatal viability no BPA-related effects on surface righting response in offspring 2250 ppm: ↓ mean pup body weight gain (PND 7-14) ↓ mean pup body weights (PND 11-21), up to 8.3% lower 750 ppm: ↓ mean pup body weight gain (PND 7-14) ↓ mean pup body weight (PND 14-17), up to 5.6% lower no BPA-related effects on post-weaning body weight
Sexual maturation	mean ages of attainment of balanopreputial separation and body weight at the age of attainment unaffected mean ages of attainment of vaginal patency and body weight at the age of attainment unaffected
Behavioural testing	detailed clinical observations: no test substance-related effects motor activity: no effects on activity counts or on pattern of activity or on pattern of habituation auditory startle: no effects on response amplitude or on the pattern of habituation learning and memory: no effects on swimming ability or on number of errors
Brain evaluations	no effects on mean brain weights, lengths, widths no histopathologic or morphometric alterations
(NOAEL for systemic toxicity: 75 ppm, approx. 5.85 and 13.1 mg/kg bw/day during gestation and lactation, respectively) (NOAEL developmental neurotoxicity: 2250 ppm approx. 164 and 410 mg/kg bw/day during gestation and lactation, respectively)	

Table C: Overview of essential components of the study of Ryan et al. 2010

<table>
<tr><td>Title of study</td><td>In Utero and Lactational Exposure to Bisphenol A, in contrast to Ethinyl Estradiol, Does not Alter Sexually Dimorphic Behavior, Puberty, Fertility and Anatomy of Female LE Rats</td></tr>
<tr><td>Authors</td><td>B.C. Ryan, A.K. Hotchkiss, K.M. Crofton, L.E Gray</td></tr>
<tr><td>Species</td><td>Long Evans rat
rat strain previously used in the lab for evaluation of estrogenic endocrine compounds and antiandrogenic compounds on reproductive development
rat strain also commonly used for the study of reproductive behaviour
female offspring of this study observed for sexually dimorphic behaviours</td></tr>
<tr><td>Treatment period</td><td>gestational day 7 (GD 7) to postnatal day 18 (PND 18)
in order to expose offspring during the period of sexual differentiation of the reproductive organs as well as the initial period of sexual differentiation of the brain

dams allowed to litter naturally</td></tr>
<tr><td>Route of exposure</td><td>oral (gavage)
route selected to simulate the most common route of exposure to humans</td></tr>
<tr><td>Dosing</td><td>bisphenol A (BPA)
concurrent positive control: Ethinyl Estradiol (EE)

<table>
<tr><td>µg/kg/d</td><td>corn oil</td><td>BPA 2</td><td>BPA 20</td><td>BPA 200</td><td>EE 0.05</td><td>EE 0.15</td><td>EE 0.5</td><td>EE 1.5</td><td>EE 5</td><td>EE 15</td><td>EE 50</td></tr>
<tr><td>Number of dams*)</td><td>38</td><td>13</td><td>20</td><td>22</td><td>19</td><td>6</td><td>35</td><td>10</td><td>37</td><td>8</td><td>24</td></tr>
</table>
*) from Howdeshell et al. 2008

study performed in two blocks</td></tr>
<tr><td>Endpoints evaluated</td><td></td></tr>
<tr><td>Maternal observations</td><td>body weight gain during pregnancy (GD 7 -20)
body weight gain during lactation (PND 2-18)</td></tr>
<tr><td>Offspring observations</td><td>sex, body weight, anogenital distance (AGD) on PND 2
sex, body weight, number of areolae/nipples on PND 14

age and weight at puberty (vaginal opening):
after weaning at PND 23: daily body weight and check for vaginal opening (VO) every other day until comple-</td></tr>
</table>

<table>
<tr><td></td><td>tion (EE 50 µg-group excluded from check for VO because of malformed genitalia)

reproductive organ morphology:
at sacrifice at approx 10 months of age: examination for abnormal external genitalia and determination of
urethral slit length (USL)
urethral slit depth (USD)
urethra-vaginal distance (UVD)
ano-vaginal distance (AVD)</td></tr>
<tr><td>Offspring fertility testing</td><td>at weaning a subset of the developmentally exposed female and male offspring were paired with an age-matched, nonsibling for at least 4 months and monitored regularly for the presence of litters

determination of fertility: if a breeding pair produced at least 1 litter, they were considered fertile
determination of fecundity: total number of pups produced</td></tr>
<tr><td>Offspring behavioural testing</td><td><u>sexually dimorphic behaviour testing</u>:

▪ evaluation of saccharin preference
[pilot study included with unexposed animals for demonstration/verification of sex-specific difference in sweet preference (females > males)]
▪ evaluation of lordosis behaviour**
[pilot study included with unexposed LE and SD female rats]
▪ evaluation of spontaneous locomotor activity levels*** in a Figure-8 maze
[pilot study included with unexposed animals for demonstration/verification of sex-specific difference in spontaneous locomotor activity level (females > males)]

** in ovariectomised females given 50 µg/kg EE for two days followed by 0.5 mg progesterone to stimulate the behaviour
*** in ovariectomised females with and without EE substitution for restoration of endogenous estrogen</td></tr>
<tr><td>Results</td><td></td></tr>
<tr><td>Maternal and pregnancy data</td><td>EE: body weight gain during pregnancy and number of uterine implantations affected :
BPA: no effects

<table>
<tr><td></td><td>BPA treated groups</td><td>EE treated groups</td><td>NOAEL/EE</td></tr>
<tr><td>Body weight gain during pregnancy</td><td>Not affected</td><td>stat. sign. ($p<0.05$) ↓ at <u>≥</u> 1.5 µg EE/kg/d</td><td>0.5 µg/kg/d</td></tr>
<tr><td>Uterine implantations</td><td>Not affected</td><td>stat. sign. ($p<0.05$) ↓ at 50 µg EE/kg/d</td><td>15 µg/kg/d</td></tr>
</table>
</td></tr>
</table>

<table>
<tr><td></td><td>.</td></tr>
<tr><td>Neonatal and pup data</td><td>EE: number and body weight of live pups, neonatal viability and AGD affected:
BPA: no effects on number and/or body weight of live pups or on neonatal viability or on AGD

<table>
<tr><td></td><td>BPA treated groups</td><td>EE treated groups</td><td>NOAEL/EE</td></tr>
<tr><td>number of live pups/litter (PND 2)</td><td>Not affected</td><td>stat. sign. (p<0.05) ↓ at ≥ 5 µg EE/kg/d</td><td>1.5 µg/kg/d</td></tr>
<tr><td>female/male pup body weight (PND 2)</td><td>Not affected</td><td>stat. sign. (p<0.05) ↓ at ≥ 50 µg EE/kg/d</td><td>15 µg/kg/d</td></tr>
<tr><td>neonatal mortality (PND 2)</td><td>Not affected</td><td>stat. sign. (p<0.05) ↓ at ≥ 50 µg EE/kg/d</td><td>15 µg/kg/d</td></tr>
<tr><td>AGD in female pups (PND 2)</td><td>Not affected</td><td>stat. sign. (p<0.05) ↓ at ≥ 50 µg EE/kg/d</td><td>15 µg/kg/d</td></tr>
<tr><td>body weight at weaning in female pups</td><td>Not affected</td><td>stat. sign. (p<0.05) ↓ at ≥ 50 µg EE/kg/d</td><td>15 µg/kg/d</td></tr>
</table>

no effects on areolae/nipples
(neither EE nor BPA induced areolae/nipple agenesis [a male rat characteristic displayed among female rats exposed in utero to androgens but not to estrogens])</td></tr>
<tr><td>Onset of puberty in F1 females</td><td>EE: exposure to EE at 0.5 and 5 µg/kg/d accelerated the age at VO (stat. sign. at p<0.01 for the 5 µg/kg/d dose group) attained at a lighter body weight
BPA: no effects on age at VO or on body weight</td></tr>
<tr><td>Fecundity and fertility</td><td>EE: stat. sign. (p<0.01) ↓ number of F2 pups produced over 4 months of continuous breeding from exposures to 5, 15, and 50 µg EE/kg/d
BPA: no effect on ability to produce viable F2 pups</td></tr>
<tr><td>Abnormalities of external genitalia in F1 females</td><td>EE: exposures to 5 and 50 µg EE/kg/d displayed (63 to 100%, respectively) malformations of the external genitalia, UVD, USL, USD altered, AVD not affected
BPA: no effects on these morphological endpoints</td></tr>
<tr><td>Behaviour in F1 females</td><td></td></tr>
<tr><td>Sweet preference</td><td>EE: exposures to 5 and 50 µg EE/kg/d: stat. sign. (p<0.01) change in sweet preference to male-like levels
BPA: no effects on this behaviour</td></tr>
<tr><td>Lordosis behaviour</td><td>EE: exposures to 15 and 50 µg EE/kg/d: stat. sign. (p<0.01) permanent change in lordosis behaviour (activation of lordosis behaviour no longer inducible)</td></tr>
</table>

	BPA: no effects on this behaviour
Spontaneous locomotor activity	Neither EE nor BPA exposure affected maze activity in ovarectomised or ovarectomised plus EE stimulated females

Table D: overview of essential constituents of studies (adapted from VMK, 2009)

Parameters	Adriani et al., 2003	Carr et al., 2003	Negishi et al., 2004	Ryan and Vanden-berg, 2006	Ryan et al. (2010)	Stump et al. (2010)	Ema et al. (2001)
Species/strain/ route	Rat/SD/oral by micropipette	Rat/Fisher/oral by gavage	Rat/Fisher/oral by gavage	Mice/C57/BL-6/oral by gavage	Rat/LE/oral by gavage	Rat/SD/oral by diet	Rat/SD/oral by ga-vage
Dose groups	Vehicle (control) arachis oil BPA in arachis oil at a concentration of 0.04 mg/kg bw/day*	Vehicle (control) saf-flower oil 0.5 ml/kg bw BPA (mg/kg bw/d) 0.10 0.25	Vehicle (control) corn oil 2 ml/kg bw BPA (mg/kg bw/d) 0.10	Vehicle (control) corn oil 0.4 µg/dose BPA (mg/kg bw/d) 0.002 0.20	Vehicle (control) corn oil 0.5 ml/kg bw BPA (mg/kg bw/day) 0.002 0.020 0.200	Control (Rodent Lab Diet 5002) BPA intake (mg/kg bw/day) during ges-tation, resp. Lacta-tion 0.01-0.3 0.02-0.25 5.85-13.1 56.4-129 164-410	Vehicle (control) distilled water 2ml/kg bw BPA (mg/kg bw/day) 0.0002 0.002 0.020 0.200
Concurrent positive control	Not performed	17 beta-estradiol 0.072 mg/kg bw/d	Nonylphenol NP 0.10 mg/kg/d 10.0 mg/kg/d	Ethinyl estradiol 5.0 µg/kg/d	Ethinyl estradiol (0.05 µg/kg/d 0.15 0.5 1.5 5.0 15.0 50.0	Not performed	Not performed

Dosing period	GD 0 - PND 25 (mating to weaning)	PND 1 – PND 14 (directly to pups)	GD 3 – PND 20	GD 3 – PND 21	GD 0 – PND 18	GD 0 – PND 21 (mating to weaning)	P generation prior to mating, through mating, gestation and lactation F1 (starting PND 23) for 10 weeks prior to mating
Number (N) of dams	9/group	Not given – presumably 10 totally	10 – 11/group	Probably 14 - 16/group	6-38/group	24/group	25/group
Offspring exposed	*In utero* and through lactation	Directly	*In utero* and through lactation	*In utero* and through lactation	In utero and through lactation	In utero and through lactation	In utero and through lactation
Reproductive and developmental parameters							
	Not performed	Not performed	Body wt of dams and pups Pathological exams of dams and pups	At weaning: AGD, body wt of pups, litter size Assessment of puberty	In female pups: AGD at PND 2 Body wt at PND 2 and at weaning	Body wt of pups: PND 1, 4, 11, 17, 21 and at weekly intervals thereafter, assessment of puberty	AGD: PND 0, 4, 7, 14, 21 Body wt of pups: PND 0, 4, 7, 14 and 21 Assessment of developmental landmarks
					Assessment of female puberty, Assessment of female reproductive organ morphology at 10 months of age		Assessment of puberty
Behavioural tests							

Offspring tested	1m and 1f per litter Totally: 9/sex/group	All exposure groups within the same litter Totally:10/sex/group	1m per litter Totally: 7-10 males/group	1ovarectomised female per litter Totally: 14-16 females/group			
Reflex ontogeny	Not performed	Not performed	Not performed	Not performed		Surface righting reflex in all available pups beginning PND 2	In F1and in F2 offspring (1m and 1f / litter, respectively) surface righting reflex beginning PND 6, negative geotaxis beginning PND 7, mid-air righting beginning PND 13
Auditory startle response						at PND 20 and PND 60 (1m and 1f per litter) Totally: 23-24/sex/group	

Activity	Not performed	Not performed	▪ open field behaviour (PNW 8) ▪ spontaneous motor activity (PNW 12)	Not performed	Spontaneous locomotor activity in Figure-8 maze (photocell counts): ovariectomised females with and without oral EE priming Totally: 23-26 females/BPA dose group	Spontaneous locomotor activity in a rectangular cage (photocell counts) PND 13,17, 21 and 61 1 m and 1f per litter Totally: 23-24/sex/group	open field behaviour (postnatal weeks 5-6) (ambulation, rearing, grooming, urination, defecation) in <u>all</u> F1 animals
Anxiety-related behaviour	Novelty preference test (PND 35-45)	Not performed	Elevated plus-maze test (PNW 14)	Elevated plus-maze test (PND 42) Light/dark preference chamber			
Learning abilities and memory	<u>Impulsive behaviour (PND >70):</u> ▪ Schedule controlled test (nose poking holes, increased delay in food delivery)	<u>Spatial memory:</u> ▪ Swim channel test (on PND 33) ▪ Morris water Maze test (on PND 34)	<u>Learning and memory:</u> ▪ Passive avoidance test (PNW 13) ▪ Active voidance test (PNW 15)	<u>Short-time spatial memory:</u> ▪ Radial-arm maze ▪ Barnes maze		Swimming ability and spatial memory in water-filled multiple T-Maze (PND 22 and 62) 1m and 1f per litter Totally: 23-24/sex/group	Spatial memory in water-filled multiple T-Maze (PND 6-7) (elapsed time or errors) 6/sex/group in F1

Pharmacological challenge	Open field response to amphetamine challenge (PND>70)	Not performed	Open field response to tcy-challenge (Monoamine disruption test)	Not performed			
Saccharin preference					Totally: 8 - 15 females/BPA dose group		
Lordosis behaviour					ovariectomised females with and without oral EE priming Totally: 6 -15 females/BPA dose group		