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Does the food processing contaminant acrylamide cause developmental neurotoxicity? A review and identification of knowledge gaps

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ABSTRACT

There is a worldwide concern on adverse health effects of dietary exposure to acrylamide (AA) due to its presence in commonly consumed foods. AA is formed when carbohydrate rich foods containing asparagine and reducing sugars are prepared at high temperatures and low moisture conditions. Upon oral intake, AA is rapidly absorbed and distributed to all organs. AA is a known human neurotoxicant that can reach the developing foetus *via* placental transfer and breast milk. Although adverse neurodevelopmental effects have been observed after prenatal AA exposure in rodents, adverse effects of AA on the developing brain has so far not been studied in humans. However, epidemiological studies indicate that gestational exposure to AA impair foetal growth and AA exposure has been associated with reduced head circumference of the neonate. Thus, there is an urgent need for further research to elucidate whether pre- and perinatal AA exposure in humans might impair neurodevelopment and adversely affect neuronal function postnatally. Here, we review the literature with emphasis on the identification of critical knowledge gaps in relation to neurodevelopmental toxicity of AA and its mode of action and we suggest research strategies to close these gaps to better protect the unborn child.

1. Background

Acrylamide (AA) is a low molecular weight, highly water-soluble substituted alkene that has been produced at a large scale for many years. It is widely used for production of organic chemicals as an intermediate and as a monomer to produce polyacrylamide – a water coagulant flocculant that can contain traces of AA monomers [1,2]. In addition, AA is used in the cosmetic and textile industries, for laboratory purposes, and as soil conditioner for wastewater treatment [3–5].

Concerns about exposure to AA of the general population arose at the turn of the century with the discovery that AA is formed in carbohydrate-rich foods containing asparagine and reducing sugars when prepared at temperatures mostly above 120 °C and low moisture [6,7]. Typically, AA is found in French fries (mean 308 μg/kg), potato crisps (mean 389 μg/kg), bread (mean 42 μg/kg), biscuits (mean 265 μg/kg) and coffee (mean 522 μg/kg dry coffee) but high levels of AA are also known to be present in cigarette smoke (497 to 169 ng per cigarette) [1,8]. Since AA is formed during food preparation there is wide-spread human exposure. Because AA is classified as a probable human carcinogen (Group 2A) by the International Agency for research on Cancer (IARC) [9], the finding that AA is present in food and drinks initiated a vast number of experimental and epidemiological studies to characterize human exposure as well as DNA adduct formation, mutagenicity and toxicity.

In 2015 EFSA Panel on Contaminants in the Food Chain (CONTAM) assessed the cancer risk and concluded that the margin of exposure (MOE) for the mean exposure from food was in the range of 283 to 50. This MOE was considered a concern as it is substantially less than a MOE of >10 000 that would be of low concern from a public health point of view [1]. In addition, AA-induced neurotoxic adverse effects have been well documented in occupational studies [10], where workers have been predominantly exposed *via* inhalational and/or absorption through the

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skin. Occupational exposure has been shown to give rise to cumulative neurotoxicity [11], and peripheral neuropathy has been shown in workers as well as in numerous experimental animal studies as summarized by the European Food Safety Authority (EFSA) in 2015 [1]. Neurotoxic effects were regarded as the most sensitive non-neoplastic endpoint with small MOEs particularly for toddlers and other children [1].

Foetal, embryonic and childhood are the major windows that are particularly sensitive to chemical exposure [12,13]. Although AA is known to reach the foetus and has been detected in breast milk [14,15], potential adverse neurodevelopment after early life exposure has so far not been studied in humans. With this in mind, the need for assessing the risk of impaired neurodevelopment associated with exposure *via* food is pertinent as a background for prioritization of risk reduction options.

In the chapters below, dietary exposure and toxicokinetics of AA including kinetic modelling are summarized, since knowledge about real-life exposure, metabolic bioactivation and potential distribution to the breast milk and the foetus are important aspects for understanding the risk whether neurodevelopmental toxicity appears after pre- and perinatal exposure to AA. We further shortly summarise existing literature on adult neurotoxicity (occupational and cohort studies) that can be linked to AA exposure in humans since this may have relevance to mechanisms of developmental neurotoxicity. The main focus is on neurodevelopmental effects *in vivo* and *in vitro* including proposed modes of action of AA and glycidamide (GA). We end this review by identifying knowledge gaps and research needs for risk characterization of developmental neurotoxicity (DNT) after pre- and perinatal exposure to AA.

2. Human dietary exposure of acrylamide

The daily dietary exposure of adolescents, adults, elderly and very elderly to AA was estimated by EFSA to be on average between 0.4 and 0.9 μg/kg body weight (bw)/day [1]. Among pregnant women in The Norwegian mother, father and child cohort (MoBa) and NewGeneris cohort, mean exposure estimates were between 0.4 and 0.6 μg/kg bw/day, and the 95th percentile estimates were between 0.6 and 1.1 μg/kg bw/day, which were in the same range as those estimated in the adult population groups representing the general population by EFSA [16,17]. Chronic dietary exposure of infants, toddlers and other children to AA was estimated to be on average between 0.5 and 1.9 μg/kg bw/day by EFSA. Children may be exposed to up to three times more AA than adults per kg bw, which can be explained by a higher energy intake/kg bw than for adults [1,18,19]. The higher exposure in children than in adults makes it particularly important to assess the potential pre- and postnatal neurodevelopmental effects.

The human safety of AA in foods has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [2]. MOEs were calculated at intakes of 1 μg AA/kg bw/day, to represent the average intake of the general population, and 4 μg AA/kg bw/day to represent the intake by high consumers. Comparison of these intakes with the NOEL of 200 μg/kg bw/day for morphological changes in nerves detected in rats by electron microscopy would provide MOEs of 200 and 50, respectively. The lower the MOE the greater the health concern. Comparison of the selected intakes with the NOEL of 2.0 mg/kg bw/day for reproductive, developmental, and other non-neoplastic effects in rodents would provide margins of exposure of 2000 and 500, respectively. The corresponding values for mammary tumours in rats were 300 and 75. Based on these MOEs, the Committee concluded that morphological changes in nerves can not be excluded for some individuals with very high intake. From all data available, the EFSA CONTAM Panel [1] stated that the data from human studies were not adequate for dose-response assessment. Therefore, the CONTAM Panel considered the data from studies on experimental animals to establish the reference points. Based on these analyses, the Panel conclude that the MOEs for the 95th percentile upper bound exposure estimates for toddlers and other children are close to the value that might be of concern for neurotoxicity [1]. These MOE calculations were based on neurotoxicity in adults and not on neurodevelopmental endpoints; the latter would possibly result in lower MoEs.

3. Toxicokinetics

3.1. Absorption and distribution

AA can be absorbed orally, dermally, and by inhalation both in humans and laboratory animals [20,21]. Studies in various mammalian species have indicated that AA is rapidly and almost completely absorbed from the GI tract. Due to its high aqueous solubility and distribution in the total body water volume, AA is widely distributed into tissues. Administration of ^{14}C-AA in male and pregnant female mice resulted in distribution into several organs including the foetal brain which was as heavily labelled as the maternal brain [22].

3.2. Metabolism to glycidamide

In vivo, AA is metabolized to a reactive epoxide GA by the liver cytochrome P450 enzyme CYP2E1. GA can also be present in foods, but at much lower concentrations than AA. Processed potato samples contained 0.3–1.5 μg GA/kg depending on processing conditions, whereas the same samples contained 200−350 μg AA/kg [23]. CYP2E1 mRNA and protein are highly expressed in the liver and only weakly in other tissues including the brain, where the highest RNA expression levels are reported in cerebellum and cerebral cortex in humans [24]. GA formed in the liver seems to be sufficiently stable to be distributed to extrahepatic tissues including the brain. Although extrahepatic expression of CYP2E1 is low and plays a negligible role in the systemic conversion of AA to GA, local conversion of AA to GA may still occur due to endogenous and inducible CYP2E1 activity. CYP2E1 is inducible in brain tissue by exposures to cigarette smoke, alcohol consumption and high fat diet, and it is elevated in several disease states [25–,26,27,28]. AA itself induced CYP2E1 expression in cultured human HepG2 cells [29] and in spermatocytes of adult mice [30].

Species differences in the expression of CYP2E1 have been reported. In rat liver, CYP2E1 expression begins within one day after birth, whereas human foetal liver at age 23–40 weeks exhibits about 1 % of the expression of adult liver [31]. Rates of formation of GA in mice were reported to be 59 % at 50 mg/kg bw (mice were 6–7 weeks of age [32]), and 33 % at 50 mg/kg bw in rats (rats were 9–10 weeks of age [32]). At 3 mg/kg bw the amount of metabolites formed *via* the GA in rats was 41 % (rat body weight range was 202−212 g [33]). This shows that the conversion to GA was higher in rodents than in human male volunteers (11 % at 3 mg/kg bw, between 26 and 68 years of age [33,34]).

Both AA and GA are conjugated to glutathione (GSH), primarily mediated by glutathione-S-transferases (GSTs). The GSH conjugates are subsequently converted to mercapturic acids, which is a detoxification pathway [1].

GSTs belongs to a superfamily with extreme diversity in amino acid sequences. Human cytosolic GSTs belong to the alpha, zeta, theta, mu, pi, sigma and omega classes. It is not clear, which GSTs are involved in the conjugation reactions of AA and GA and it has been suggested that isoforms of the GSTP and/or GSTT families may protect mice from acute AA neurotoxicity [35]. Polymorphisms are described for several GSTs, and the best studied polymorphisms are for GST mu, theta and pi. For GST mu and theta, a null phenotype is described resulting in complete loss in activity of these GSTs. In one study [36], individuals with null variants of GSTM1 (mu morphism) and GSTT1 (theta morphism) had a higher ratio of GA to AA conjugated to haemoglobin (Hb) in their blood than those with the wild-type genotypes indicating reduced rate of AA detoxification [36]. GA, like AA, is widely distributed to tissues including the brain [37]. The elimination half-life of AA based on urinary excretion in humans was found to be 3.1–3.5 h [34]. The estimated

plasma half-life in male Sprague Dawley rats was approximately 2 h for both AA and GA [38].

Numerous studies have demonstrated that both AA and GA react with cysteine, but also at terminal valine amino acids in Hb, forming the adducts AA-Hb and GA-Hb in red blood cells. The presence of these adducts are often used as biomarkers of exposure together with excreted mercapturic acid derivatives of AA and GA. AA and GA may also react with other proteins including plasma proteins [39], and GA also reacts with nucleic acids. Covalent adducts of GA with DNA have been demonstrated *in vivo* in animal models and *in vitro* [40–42], however, AA-DNA adducts have not been observed *in vitro*, or *in vivo* in animal or human tissues [43].

In summary, due to its high liquid solubility and distribution in the total body water, AA is freely distributed into all organs. AA is bioactivated by CYP2E1 by epoxidation to GA, which is reactive towards DNA. The main metabolic inactivation reactions comprise formation of GSH adducts of AA and GA, and hydrolysis of GA; the AA metabolites are rapidly and almost completely excreted in urine. There are no reports on bioaccumulation of AA, although residual protein adduct formation may occur.

3.3. Foetal exposure

Results from the NewGeneris project that used biochemical and cytogenetic markers to investigate prenatal exposure of dietary carcinogens showed that AA readily crosses the placental barrier and that dietary exposure to AA can be detected in the foetus as AA-Hb adducts in cord blood [44,45]. Sorgelet and co-workers reported that transfer of AA through human placenta took place in an *ex vivo* model [14]. The maternal side of three post-partum human placentas was perfused with AA without recirculation of the perfusate and AA was measured on the infant's side after 5−30 min of perfusion. In another *ex vivo* study, a dual recirculating human placental perfusion was used and the transfer rate of AA (at maternal concentrations of 5 and 10 μg/mL) and GA (5 μg/mL) through the placenta was determined [46]. AA and GA crossed the placenta from the maternal to the foetal side, and the concentrations of AA and GA in the maternal and foetal circulation equilibrated within two hours [46]. In other *ex vivo* perfusion studies with human placentas, AA and GA were both found to exhibit a high placental transfer [47]. Trans-placental exposure has also been shown to occur in pregnant women where AA-Hb adducts (biomarker of exposure) could be found in blood samples of the mothers and neonates [48]. The mean ratio of AA-Hb in cord blood to maternal blood was 0.48 in a study involving 219 neonates and 87 mothers from Denmark [15]. A highly significant correlation was observed between cord blood and maternal blood for GA-Hb, where the adduct ratio of cord to maternal blood was around 0.38. Furthermore, in support of these studies, Pedersen and colleagues reported that the median AA-Hb adduct levels in cord blood were approximately half of the levels in paired maternal blood sampled from Greece, Spain, England, Denmark and Norway [17]. Hb adduct levels in cord blood were positively correlated with both maternal AA-Hb adduct and GA-Hb adducts. Intravenous injection of ^{14}C-labelled AA in pregnant rats, rabbits, beagle dogs and miniature pigs showed that radioactivity (and presumably AA or an AA metabolite) reached the foetuses [49–51].

AA can also reach the human breast milk, indicating that AA exposure may occur also *via* breastfeeding. Transfer of AA from food into human milk after consumption of potato crisps has been reported by Sorgel and colleagues [14]. The food item contained about 1 mg/kg chips of AA (approximate dosage 15 μg/kg bw) and concentrations of AA in the low μg/kg range were observed in the breast milk between 3 and 8 h after consumption [14]. In a Swedish study of non-smoking mothers (four pooled breast milk samples from 14 individuals exposed to a daily dietary AA intake of about 0.5 μg/kg bw), the concentration of AA in breast milk was found to be below the LOQ of 0.5 μg/kg, except in one individual sample (0.51 μg/kg) [52]. However, since this study consisted of a limited number of breast milk sample analyses of these results may not be representative for pregnant women in general.

Overall, the above publications show that the *in vivo* levels of AA and GA in foetal and maternal blood are about the same and that the placenta provides limited protection of the foetus to exposure from these compounds if present in the maternal blood. Additionally, infants may be exposed to AA through breast milk.

3.4. Physiologically based toxicokinetic models

Several studies have reported various approaches to physiologically based toxicokinetic (PBTK) modelling of AA and GA absorption, metabolism, and distribution [53–55] with the goal of predicting human internal exposures to AA and GA (*i.e.* area under the curve, AUC) to reduce the uncertainty when extrapolating results from animals to humans in risk assessment. PBTK models allow derivation of human-equivalent doses (HED) based on the AUCs of AA and GA for the same dose in humans and animals. The HED can be used to convert the external critical effect doses from animal studies to humans. In 2003, Kirman et al. developed a PBTK model for AA and GA in rat that included five compartments. The model also accounted for metabolism, Hb adduct formation and binding to other macromolecules [53]. Walker et al. modified the model and assessed AA and GA dosimetry in rats and human adults and children. The adult human model was scaled to fit children's physiology (age 0–1 year) and accounted for the immature metabolic GST/GSH detoxification pathway in children. The estimates of child/adult dosimetry differences in AUC was shown to be modest (two- to five-fold) when considering the 99th percentile (child) to median (adult) values [56]. Young and collaborators developed a model, focusing on the toxicodynamic process of AA and GA Hb adduct formation and GA-DNA binding in liver for rat, mice and humans [55]. In 2008, they used the model to integrate rodent neurotoxicity findings into human extrapolation. The model did not suggest a risk of human neurotoxicity due to dietary AA exposures. This is due to the high dose administered to rodents not being relevant for human daily dietary exposure [57]. In 2010, Sweeny and colleagues expanded the model to nine compartments and applied it for both rats and humans [54]. This model was used by DeWoskin et al. 2013 to compare internal dosimetry for AA and GA as AUCs [58].

An *in vitro* to *in vivo* extrapolation (IVIVE) of AA toxicity was performed in 1999 by DeJongh et al. [59]. Neurotoxic effects of AA were studied *in vitro* by exposure of the human neuroblastoma SH-SY5Y cell line for 72 h. The test battery studied various endpoints for cell physiology, morphology and neurochemistry in differentiated cells. The lowest concentration of AA that induced 20 % effect (EC_{20}) was assumed to be equivalent to the target tissue concentration and was integrated in the PBTK model. Hence, the EC_{20} value was used as a surrogate for the lowest observed effect level (LOEL) for neurotoxicity. A one-compartment kinetic model for rat was developed to estimate acute and sub-chronic toxicity of AA *in vivo*. For the IVIVE, the PBTK model simulation was used to estimate the Lowest Observed Effective Dose (LOED) that would result in the target concentration equivalent to the LOEL. A generally good agreement was seen between the estimated and experimental LOEDs in rat [59].

None of the PBTK models summarized above have a foetal compartment included. Therefore, more knowledge on the distribution of AA and GA into the foetal brain by further development of the PBTK models including a foetal compartment would improve human risk assessment of AA.

4. Neurotoxicity

Numerous studies in experimental animals and observations in humans in occupational settings show that both the peripheral and the central nervous system are principle targets for AA toxicity.

4.1. Adult human cohort studies on neurotoxicity

AA exposure can cause neurological symptoms in humans after exposure through inhalation, ingestion (diet) and skin absorption. The variety of reported signs of neurotoxicity show involvement of the central (CNS) and the peripheral nervous system, as well as the autonomic nervous system [60]. The neurotoxic effects of AA are progressive both in the peripheral nervous system and the CNS indicating that the damages are accumulating. Hence, repeated exposure to AA may start with mild symptoms, progressing into severe disability with irreversible damages [61]. In contrast to the general population were diet is the major source of exposure, workers are predominantly exposed *via* inhalational and/or the skin.

Although many cases of AA poisoning have been reported in the literature following occupational exposure [62], only two human population studies were identified on the possible association between dietary AA intake and neurological symptoms [63,64]. In these two studies, the reported effects were hearing loss and a mild cognitive decline and an increased risk of poor cognition. Supplementary Table 1 summarizes the cohort and occupational studies of AA induced neurotoxicity in adults, including number of workers, gender, exposure levels and duration, and health outcomes.

4.2. Pre- and perinatal acrylamide exposure and developmental human effects

Normal human brain development starts in the second week of pregnancy and continues through puberty. It is a complex process with both time-dependent and spatial patterning [74]. Particular concern has been expressed over the impact of toxicants on brain development and possibly neurodevelopmental disorders originating in the prenatal period. Brain development involves processes like neuronal proliferation, commitment of neuronal and glial progenitor cells followed by migration, differentiation into neuronal and glial cell subtypes, synaptogenesis, pruning, myelination, network formation and terminal functional neuronal and glial cell maturation [75–81].

It is well documented from experimental studies that during critical periods of brain development, even low exposure to toxicants can disrupt processes involved in brain development [77], potentially leading to adverse effects. Whereas we found no studies in literature on prenatal exposure to AA and neurodevelopmental (cognitive, language development *etc.*) effects in children, there are a few cohort studies in humans showing associations between prenatal dietary AA exposure *via* the maternal diet and reduced foetal growth including reduced head circumference. As these effects are relevant to foetal AA exposure and possibility of neurodevelopmental effects not yet studied, they are shortly reviewed here.

In the prospective NewGeneris European mother-child study of 1101 mother-child pairs across Europe, mothers with a diet rich in AA had higher levels of AA-Hb and GA-Hb adducts in umbilical cord blood, and there was a negative association between the levels of these adducts and birth weight and head circumference [17].

Two studies from MoBa examined prenatal AA exposure in approximately 50,000 mother-child pairs based on maternal intake obtained from validated food frequency questionnaires combined with data on AA concentrations in food. In the first study, they found that AA intake during pregnancy was negatively associated with foetal growth, measured as reduced birthweight and small for gestational age [16]. In the second study postnatal growth in children at age 3, 5 and 8 years was investigated. Children born to mothers with the highest AA intake in pregnancy showed a moderately increased prevalence of overweight/-obesity compared to peers that had the lowest prenatal AA exposure [82]. The association between maternal AA intake and child overweight/obesity was not modified by the child's own intake of AA, suggesting that prenatal exposure to AA may be more important.

In the French EDEN mother-child cohort the association between prenatal AA exposure as estimated by dietary AA intake during pregnancy and offspring anthropometry was examined. The study population consisted of 1471 mother-child pairs. They found that an increased intake of dietary AA was associated with small for gestational age and decreasing birthweight [83]. These studies add to the evidence that negative effects on foetal growth may even occur at low level dietary AA exposure.

A recent Japanese study of 204 mothers that gave birth to girls showed that maternal AA intake during pregnancy was positively associated with higher levels of umbilical cord blood levels of the sex hormone oestradiol, but not with hormone levels in maternal blood. They also found a marginal positive association between AA intake and head circumference [84].

In a recent meta-analysis [85] on the relationship between gestational AA exposure and offspring's growth, five cohort studies with 54, 728 participants were included. Among the included studies were the two from Norway [16,82], the French EDEN study [83], a combined European study [47] and the Japanese study [84] all described above. The authors found an association both between gestational AA from maternal dietary exposure and reduced birth weight and an increased risk for small for gestational age. In support of these data, reduced foetal growth following gestational AA exposure has been observed in animal experiments [86]. Also, a higher risk of developing overweight or obesity later in life was suggested by Zhan and collaborators [85].

Smoking is a major source of human AA exposure [87,88]. It is well known that smoking may affect neurodevelopment, however, it is not known whether AA, in addition to nicotine or other compounds in cigarette smoke, might contribute to these effects. The impact on growth from maternal dietary AA exposure was similar in smoking and non-smoking women. In one of the two studies, AA exposure was estimated by measuring AA-Hb adducts, and the association between AA exposure and birth weight was present as well. In both studies the possibility of residual confounding by cigarette smoking was adequately addressed by stratified analyses according to smoking status [16,17].

It is well known that foetal growth restriction is associated with adverse neurodevelopment in children, shown as structural brain alterations (*e.g.* reduced total brain and cortical volume, decreased total number of cells) in addition to problems in motor skills, cognition, memory and neuropsychological dysfunctions [89]. However, it is still not shown whether the far less pronounced birth weights loss shown after pre-natal AA exposure adversely affect neural function postnatally.

Based on existing experimental evidence and the clear indication from human studies that gestational exposure to AA may impair growth of the foetus and reduce head circumference, we conclude that there is an urgent need for further research to examine whether pre- and perinatal AA exposure might impair neurodevelopment in humans.

4.3. Hippocampal neurogenesis in adult animal models

Adult neurogenesis seems to be restricted to the hippocampus [90,91]. Like the developing brain of the unborn child, also adult hippocampal neurogenesis includes processes like stem cell proliferation, differentiation of progenitor cells, migration of new-born neurons, synaptic growth and axon formation of pyramidal cells in the cornus ammonis 3 zone (CA3) [92–95] and has therefore been included in this review. A few animal studies show that AA may cause behavioural deficits in adults, where some of the effects may be attributed to hippocampal function. Adult hippocampal neurogenesis is a multistep process involved in cognitive functions considered to be essential for humans [96]. Microglia may also have an important role in adult neurogenesis, as they can sense subtle changes in their environment and may use this information to modulate the production of new neurons in the adult hippocampus [97]. The studies presented below on effect of AA in adult rodents may thus point to mechanisms of actions that are of relevance also for the developing brain. In a recent study, AA exposure (10 mg/kg bw/day for 7 weeks) was associated with an activation of

glial cells [98]. In another study, male mice were orally administered with low doses of AA (0.002, 0.02, or 0.2 mg/kg bw/day for 4 weeks). Hippocampal neurogenesis and neurocognitive functions were adversely affected at 0.2 mg AA/kg bw/day [99]. This low effect level (LOEL) is the same as the no observed effect level (NOEL) of 0.2 mg/kg bw/day by JECFA [2] based on morphological examination by electron microscopy of peripheral nerves in adult rats [100]. Hence, hippocampal neurogenesis seems to be more susceptible to AA than peripheral nerves. In male 10-week-old rats fed with 0.2−20 mg/kg bw/day of AA for 5 weeks, microglial activation (at 2 mg/kg bw) was demonstrated by increased expression of microglial markers (CD11b and CD40) in the cerebral cortex [101]. A similar study with rats fed with 0.5−5 mg/kg bw/day for 12 months showed microglial activation in the hippocampus and frontal cortex [102]. The increased expression of the potent proinflammatory cytokine IL-1β was observed already at the lowest dose (0.5 mg/kg bw/day) [102]. Furthermore, others reported toxicity of AA towards hippocampal neurogenesis where 50 mg/kg bw/day for 2 weeks was found to significantly decrease the number of newly generated cells in the dentate gyrus in mice [103]. Several studies thus suggest an impairment of adult neurogenesis in response to AA exposure with effect being observable at relatively low doses (BMDL10 of 0.43 mg/kg bw/day).

4.4. Neurodevelopmental effects in experimental animal models

Although numerous studies have examined the neurotoxicity of AA in adult animals, much less is known about its effects on neurodevelopment after pre- and postnatal exposure. As shown in Table 1, developmental studies in animal models show signs of neurodevelopmental toxicity as well as histological changes in the CNS. Neurobehavioral effects are observed at exposure levels that are in some cases also associated with maternal toxicity (including neurotoxicity and decreased maternal body weight) and in these cases the potential direct effect of AA on offspring neurodevelopment may be difficult to establish.

4.4.1. Histopathological and molecular alterations

The first indication that AA could cause neurodevelopmental toxicity was in 1995, when Wise and colleagues reported that maternal AA exposure starting at 15 mg/kg bw/day significantly decreased average horizontal motor activity and auditory startle response in Sprague-Dawley rat pups [104]. The AA dose was additionally seen to cause neurotoxicity in the rat dams who showed hindlimb splaying. Other studies have since then reported alterations in neurodevelopment after AA exposure, which has mainly been investigated in rats. Maternal AA exposure of 30 mg/kg bw/day in rats during pregnancy has resulted in decreased brain weight and decreased number of cerebellar Purkinje cells and internal granular layers in the pups [105]. Ultrastructural analysis of the Purkinje cells revealed changes in the endoplasmic reticulum and loss of normal arrangement of polyribosomes, swollen mitochondria with abnormal differentiated cristae as well as abnormal Golgi apparatus. Exposure from gestational day (GD) 7 to postnatal day (PND) 21 with a low AA dose of 10 mg/kg bw/day administered to the dams, resulted in a lower number of Purkinje cells and a decreased cerebellar weight [106]. It was also observed that the pups had decreased volume of granular and molecular layer and increased volume of white matter, which indicated that AA induced structural changes in the development of the cerebellar cortical layers. Maternal AA dose of 10 mg/kg bw/day has additionally been reported to cause motor neuron degeneration, myelin degeneration, neurofilament reduction, induce apoptosis as well as reactive gliosis at PND28 in rat offspring [107]. AA has also been observed to increase the number of GABAergic neurons in rat pups at a maternal AA dose of 50 mg/l (given in the drinking water), which was indicated by increased glutamic acid decarboxylase 67-immunoreactive cells in hippocampus [108]. The same study further reported that AA starting at 25 mg/l dose-dependently increased the number of reelin immunoreactive cells, where reelin is a molecule regulating neuronal migration and positioning in the hilus of the hippocampal dentate gyrus. Furthermore, AA exposure at 100 mg/l has been reported to decrease progenitor cell proliferation in the subgranular zone (SGZ) as well as decrease apoptosis [108,109], where the SGZ cell proliferation and reelin-producing interneurons density were seen recovered at PND77. Reduced number of neurons has been reported at PND21 after maternal AA exposure to 10 and 20 mg/kg bw/day, with reduced expression of growth associated protein 43 (GAP43) and reduced level of synaptophysin in the pups, suggesting toxic effect on the development of hippocampal neurons [95]. Long-term exposure to 3.0 mg/kg bw/day of AA, starting *in utero* until 2 years of age, increased the incidence of spinal cord degeneration in the rat pups as well as increased gliosis and sciatic nerve neuropathy [110]. Investigating the toxic effect on the molecular level and behavioural endpoints has revealed that 100 ppm AA-exposure in Wistar rat dams exposed during GD6 to PND21 in drinking water changed the activity of antioxidant enzymes in the brain of the offspring, with elevated levels of nitric oxide in the cerebellum and behavioural disturbances in the open field test and the elevated plus maze, suggesting anxiogenic responses [111].

Other studies have reported structural brain changes in the offspring due to maternal AA exposure. Histological analysis of foetal brain tissue from Wistar rats at GD20 exposed to a maternal AA dose of 5 mg/kg bw/day showed degeneration in neuronal structures and increased haemorrhagic damages, with decreased brain derived neurotrophic factor (BDNF) levels and increased malondialdehyde (MDA) [112]. Peripheral nerves in rat pups exposed to a maternal AA dose of 5 mg/kg bw/day have further exhibited axonal fragmentation and/or swelling [113]. Necrotic death and haemorrhagic damage in foetal brain tissue have also been reported at a maternal AA dose starting at 25 mg/kg bw/day, with decreased BDNF levels and induced oxidative stress [114]. Oxidative stress was indicated by increased MDA and superoxide dismutase (SOD) levels in the study, as well as reduced levels of glutathione peroxidase and catalase (CAT). Oxidative stress in the brain of rat offspring has been observed after maternal AA dose of 200 ppm (given in the drinking water from GD6-GD19) [115], and *via* maternal AA dose of 10 mg/kg bw/day, with significant reduction in GSH, total thiols, SOD and peroxidase activity in developing cerebellum [116]. In the latter study, AA was reported to further delay proliferation, cell migration and differentiation in the granular layer as well as induce loss of Purkinje cells. AA dose of 10 mg/kg bw/day in pregnant dams (from GD7 till birth) has also been reported to increase lipid peroxidation and suppress the antioxidant defence system in the medulla oblongata in rat pups [117].

Postnatal maternal AA exposure has been reported to alter neurotransmitter levels in rat pups, where maternal AA exposure of 25 mg/kg bw/day during the suckling period (until day 21) resulted in decreased levels of noradrenaline, dopamine (DA) and serotonin (5-HT) [118]. Pups that were exposed orally to 25 mg AA /kg bw/day for 5 consecutive days at PND12−21 were more sensitive compared to pups that were exposed to AA for 5 consecutive days at an age of 30 or 60 days respectively, where no change in neurotransmitter levels was reported [118]. The increased sensitivity in the younger verses older animals were presumably due to the ongoing brain neurogenesis and the still developing blood brain barrier in the PND 12–21 pups.

Younger (4, 8 or 15 days old) rats in comparison to adults exhibited a greater change in the levels of DA or 5-HT. Contradictory to these results, maternal AA dose of 25 mg/kg bw/day has also been reported to cause toxicity in the mother, where the observed effect on the offspring during lactation were consistent with inanition from maternal toxicity and the pups exhibited recovery post weaning [119].

An AA dose of 20 mg/kg bw/day in Fischer rat dams from GD7 to GD16 also gave transient effects, where 2-weeks old pups displayed decreased DA receptor affinity (Kd) and receptor site density demonstrated by decreased [^{3}H]spiroperidol binding in striatal membranes, which could not be seen at 3 weeks of age [120]. AA-dose of 15 mg/kg bw/day for 28 days administered postnatally to 21 days old rats resulted

Table 1
Experimental animal studies (mouse, rat, chicken, zebrafish) on AA developmental neurotoxicity.

Species	Dose and exposure	Neurotoxic effects	Neurodevelopmental NOAEL/LOAEL	References
Wistar rats.	100 ppm GD6 to PND21 in drinking water (no information on AA dose in mg/kg bw provided).	Changes in antioxidant enzyme activities in maternal and offspring brains, elevated nitric oxide levels in the cerebellum of the offspring, and disturbed acetylcholinesterase activity and changes in dopamine levels in the maternal cortex. Anxiogenic responses of male offspring in the elevated plus maze (less open arm entries, lower open arm duration, lower closed arm entries, and higher closed arm duration) and in the open field test (less entries into the centre and less time spent into the centre).	NOAEL not determined Maternal toxicity observed at 100 ppm.	[111]
Wistar rats.	N = 9/group: 1) Controls; 2) N-acetylcysteine (NAC) (250 mg/kg bw/day); 3) AA (25 mg/kg bw/day); 4) AA plus NAC (25 mg/kg bw/day AA and 250 mg/kg bw/day NAC, for 20 days *via* oral gavage. One foetus per litter were randomly selected for analysis of biochemical and histopathologic parameters.	AA caused necrotic death and haemorrhagic damages in foetal brain tissue with decreasing BNDF levels and increasing oxidative stress. NAC prevented the toxic effects of its on foetal brain. Oxidative stress markers were increased malondialdehyde (MDA) and SOD levels, and reduced BDNF, glutathione peroxidase and CAT levels along with its toxic effect in foetal brain.	NOAEL not determined.	[114]
Wistar rats.	20 pregnant rats were orally fed with AA 10 mg/kg bw and vitamin C 200 mg/kg bw (from GD7), 6 pups of each group were randomly selected for analysis at PND 21.	Newborns of AA-treated female rats had decreased cerebellar weight and lower than average number of Purkinje cells. AA also decreased the volume of granular and molecular layer and increased the volume of white matter. Decrease in white matter volume was observed in the vitamin C group. The authors conclude that AA induces structural changes in the development of the cerebellar cortical layers in rat newborns, and these changes may be prevented by vitamin C (antioxidant).	NOAEL not determined.	[106]
Sprague-Dawley rats.	0, 5, 10 or 20 mg/kg bw/day intragastrically, embryonic days 6−21.	Gait scores of gravid rats increased (10 mg/kg bw group), suggesting maternal motor dysfunction. Histological changes in the hippocampal neurons (all dose levels), number of neurons, expression of growth associated protein 43 (all dose levels) and synaptophysin (10 and 20 mg/kg group) reduced with increasing AA dose in PND 21 weaning rats. Authors suggest dose-dependent toxic effects on growth and development of hippocampal neurons of weaning rats.	NOAEL not determined.	[95]
Wistar rats.	Five groups (N = 8/group): control (C), corn oil (CO), vitamin E (Vit E), AA (5 mg/kg bw/day during pregnancy), and Vit E + AA. On GD20, foetuses were removed, and brain tissues examined for biochemical and histological changes.	AA caused degeneration in neuron structures in foetal brain tissue and aspartate; decreased BDNF levels; increased MDA, total oxidant capacity levels; and decreased reduced GSH and total antioxidant capacity levels. Vit E suppressed the effects of AA on foetal development and foetal brain tissue damage.	NOAEL not determined.	[112]
Sprague Dawley rats.	Rat offspring of treated female rats divided into control, rosemary; AA (10 mg/kg bw/day from GD7 to PND28); and recovery (AA and rosemary) groups.	AA caused oxidation, motor neuron degeneration, apoptosis, myelin degeneration, neurofilament reduction, and reactive gliosis.	NOAEL not determined.	[107]
Wistar rats.	Pregnant dams were given oral supplements of a combination of fructo (FOS)- and xylooligosaccharides (XOS) (FOS + XOS, 3 g/kg bw/day, GD 0−19) were exposed to AA (200 ppm in drinking water, GD6−19) (no information on AA dose in mg/kg bw provided).	AA exposed dams fed prebiotics displayed higher exploratory behaviour in the open field test. Prenatal evaluation showed that AA-induced decrements of placental/foetal weights were markedly restored with prebiotic feeding. Prebiotics significantly offset markers of oxidative stress, restored enzymatic antioxidants, cholinergic and mitochondrial function in the maternal and foetal brain. Concomitantly, prebiotics restored AA-induced depletion in the levels of dopamine and GABA in the maternal cortex that positively correlated with cecal bacterial numbers. According to the	NOAEL not determined.	[115]

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Table 1 (*continued*)

Species	Dose and exposure	Neurotoxic effects	Neurodevelopmental NOAEL/LOAEL	References
		authors, these data suggest that prenatal prebiotic oligosaccharide supplements protect developing brain against oxidative stress-mediated neurotoxicity.		
Wistar Han rats.	AA in drinking water (0, 0.5, 1.5 or 3.0 mg/kg bw/day) starting at GD6 until 2 years of age.	Increases in the incidences of spinal cord degeneration, gliosis and sciatic nerve neuropathy in male and female rats.	NOAEL 0.5 mg/kg bw/day (based on sciatic nerve neuropathy at 1.5 mg/kg bw/day in males).	[110]
Albino rats (Rattus norvegicus).	Saline (group A) or AA by gastric intubation (10 mg/kg bw/day), from GD7 till birth (prenatal intoxication, group B) or from GD7 till PND28 after birth (perinatal intoxication, group C). The pups from each group were killed on PND7, 14, 21 and 28.	Signals of AA toxicity were observed postnatally in the treated mothers (ataxia, splayed hind limbs, weakness of the hind limb muscles, and paralysis), which caused alterations in maternal behaviour. Newborns suffered from poor lactation, and consequently, malnutrition, particularly in group C. The newborns of all groups were hairless at birth. The time when fur appeared and ears and eyes opened was delayed in groups B and C. Exposure during gestation and lactation produced oxidative stress and suppression in the antioxidant defence system in the medulla oblongata of newborn rats. The lipid peroxidation level was markedly elevated, whereas the GSH and total thiol content were greatly depleted. Antioxidant enzyme activities (SOD and peroxidase) were depressed. TBARS observed in the study paralleled the decrease in the GSH concentration in the medulla oblongata of AA-treated newborns. The authors indicated that the enhanced lipid peroxidation and deterioration of the antioxidant defence system that resulted from AA exposure may play a significant role in the pathogenesis and deleterious histological effects on the medulla oblongata of newborns. The pathological cases reflected CNS neuropathy caused by AA. These effects, which appeared as histopathological changes within the medulla oblongata, resulted from perturbations of oxidative stress.	NOAEL not determined.	[117]
Zebrafish embryos, eleutheroembryos and Larvae.	AA exposure to spontaneous tail coilings in zebrafish embryos aged 24–26 h post fertilization (hpf) and the swimming activity of eleutheroembryos at 120 and larvae at 144 hpf, i.e. parameters for locomotor activity were investigated.	AA showed DNT (spontaneous tail coiling, hyperactivity). No effects on swimming activity of eleutheroembryos or larvae. DNT index (ratio LOEC mortality/LOEC locomotion for spontaneous tail coilings) was 3.99.	Frequency tail coilings LOEC: 3.52 ± 1.76 mM. Total duration tail coilings (s) LOEC: 7.03 ± 3.52 mM.	[129]
Bobcock strain chick embryo.	AA (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg, single dose administered to fertilized chick embryos (eggs) at day 8, 9 and 10 (brains collected at day 11 post fertilization).	AA caused reduced GSH and vitamin C levels from 0.1 mg AA, mild structural damages at 0.3 and 0.4 mg treatment and further mild haemorrhages, necrotic damages and formation of vacuoles were observed at 0.5 and 0.6 mg AA treatments in hippocampus region of chick embryo brain tissue.	NOAEL not determined.	[122]
Immature male and female rats (*Rattus norwegicus)*	15 mg AA/kg bw/day for 28 days.	Behavioural disturbances, increase in brain norepinephrine, glutamate, aspartate and taurine, but reduced dopamine and serotonin levels.	NOAEL not determined.	[121]
Sprague-Dawley rats.	Dams exposed to 0, 4, 20, 100 mg/l (0.36−0.89, 1.77−4.29, 8.26−19 mg/kg bw/day, respectively) in drinking water from GD10 to PND21.	No gait abnormality of dams through to the day 21 after delivery and no significant changes were observed in food intake and water intake consumption. Decreases in the body and absolute brain weights of offspring at the high dose that continued to PND77, however, gait abnormalities were not observed. On PND21, maternal AA-exposure decreased progenitor cell proliferation in the subgranular zone (SGZ) at the two highest dose levels, accompanied with increased density of reelin-producing interneurons and NeuN-expressing mature neurons within the hilus at 100 mg/L. In the SGZ of the 100 mg/l group, cellular populations immunoexpressing doublecortin or dihydropyrimidinase-like 3,	The authors considered the lowest dose level of 4 mg/l (corresponding to 0.36–0.89 mg/kg bw per day, based on water intake) to be the NOAEL. However, due to several limitations of the study, EFSA [1] did not consider the data suitable for identifying a NOAEL.	[109]

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Table 1 (*continued*)

Species	Dose and exposure	Neurotoxic effects	Neurodevelopmental NOAEL/LOAEL	References
		were decreased suggesting postmitotic immature granule cells. On PND77, the SGZ cell proliferation and reelin-producing interneuron density recovered, while the hilar mature neurons sustained to increase at the two highest dose levels.		
Albino rats (*Rattus norvegicus*).	Dams exposed to 0 or 10 mg AA/kg bw/day by gastric intubation, either from GD7 till birth (prenatal intoxicated group); or from GD7 till PND28 (perinatally intoxicated group).	Signs of AA toxicity observed postnatally on the treated mothers (ataxia, splayed hind limb, weakness of hind-limb muscles and paralysis causing alteration in maternal behaviour), therefore newborns suffered from bad lactation and consequently malnutrition. At birth, the newborns of all groups were hairless. The time of fur appearing and ear and eye opening was retarded in newborns from treated dams. Prenatally or perinatally administration induced significant retardation in body weights development of the newborn rats, and to increase thiobarbituric acid-reactive substances (TBARS) and oxidative stress (significant reductions in GSH, total thiols, SOD and peroxidase activities) in the developing cerebellum. AA treatment delayed the proliferation in the granular layer and delayed both cell migration and differentiation. AA treated animals also displayed Purkinje cell loss. Ultrastructural studies of Purkinje cells in the perinatal group showed microvacuolations and cell loss. The authors concluded that prenatal and perinatal exposure to AA caused oxidative stress, resulted in a marked suppression of the antioxidant defence system and induced structural changes in the developing rat cerebellum.	NOAEL not determined. Maternal toxicity observed at 10 mg AA/kg bw/day.	[116]
Sprague-Dawley rats.	Dams exposed to 0, 3.7, 7.9 and 14.6 mg/kg bw/day (0, 25, 50 or 100 mg/l) in drinking water from GD6 until weaning on PND21.	Dams in the 100 mg/l group exhibited gait abnormality from PND2, which progressed to a moderate or severe degree at PND21. Body weight in this group was suppressed in parallel with the progression of neurotoxic symptoms. At 50 mg/L, a slightly abnormal gait appeared from PND18. No apparent abnormalities were found on clinical observation in offspring exposed to AA maternally at any dose. Maternally exposed offspring showed decreased body weight at 100 mg/l (nearly 50 %), increased dose-dependently the number of Reelin-immunoreactive cells (a molecule regulating neuronal migration and positioning in the hilus of the hippocampal dentate gyrus) (from 25 mg/l AA) and glutamic acid decarboxylase 67-immunoreactive cells (from 50 mg/l AA), confirming an increase in GABAergic interneurons. The results revealed decreased apoptosis in the neuroblast-producing subgranular zone of the dentate gyrus of maternally exposed pups at 100 mg/l.	LOAEL was 3.72 mg/kg bw/day. Maternal toxicity observed.	[108]
Albino rats (*Rattus norwegius*).	30 mg/kg bw/day during pregnancy, or fed a standard diet (control).	Delayed growth and decreased body and brain weights. Light microscopic studies of the cerebellar cortex revealed decreases in Purkinje cells and internal granular layers. Pups showed different patterns of cell death in Purkinje cells and neurons in the brain. Ultrastructural analysis of Purkinje cells revealed changes in the endoplasmic reticulum, loss of the normal arrangement of polyribosomes, swollen mitochondria with abnormally differentiated cristae, and an abnormal Golgi apparatus. The gastrocnemius muscle in the AA group showed extensive degeneration of myofibrils as evidenced by poorly differentiated A, H, and Z bands. Authors	NOAEL not determined.	[105]

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Table 1 (*continued*)

Species	Dose and exposure	Neurotoxic effects	Neurodevelopmental NOAEL/LOAEL	References
		conclude that rat foetal exposure to AA *via* dosing pregnant dams at a dose level of 30 mg/kg bw per day, causes cerebellar cortical defects and myodegeneration of the gastrocnemius muscle during the postnatal development of pups.		
F344 rats.	Pups exposed daily beginning prenatally and throughout the lifespan. Dams were gavaged from GD6 onwards (0, 0.1, 0.3, 1.0 or 5.0 mg/kg bw/day) through parturition. Pups same dose levels by gavage through weaning until PND22 after which dosing continued *via* their drinking water.	Altered performance in an incremental repeat acquisition (IRA) task to assess learning ability by 4 months of age. From approximately 1–8 months of age (through ~ PND240), over 52 testing sessions, a significant treatment effect was found on per cent task completed (PTC), with a significantly lower PTC for the 5.0 mg/kg bw per day group compared to controls. While there was no treatment effect on accuracy, a significant decrease in response rate was seen at 5.0 mg/kg bw per day.	NOAEL of 1.0 mg/kg bw per day (however, data on IRA response, from which the NOAEL was derived, revealed only a reduction at the highest dose level tested which made the data not suitable for dose-response modelling, according to [1].	[128]
F344 rats.	0.0, 0.1, 0.3, 1.0 or 5.0 mg AA/kg bw/day by gavage (GD6 and ending on the day of parturition). Beginning on PND1 and continuing through PND21, all pups/litter were gavaged with the same dose as their dam.	No effects in offspring on parameters including fur development, pinnae detachment or eye opening. Offspring body weight was somewhat decreased in the 5.0 mg/kg bw per day group, particularly in males. AA treatment did not significantly alter righting reflex (PNDs 4–7), slant board (i.e. negative geotaxis) (PNDs 8–10), forelimb hang (PNDs 12–16), and rotarod behaviour (PNDs 21–22). Male and female offspring of the 5.0 mg/kg bw per day group were 30–49 % less active in the open field at PNDs 19–20. Comparable serum AA levels of GD20 dams and their foetuses indicating that AA is able to cross the placental barrier. The authors concluded that overt preweaning neurobehavioral effects are apparent in rats exposed to AA pre- and postnatally.	A NOAEL of 1.0 mg/kg bw/day was identified by [1] (the data on offspring body weight, from which the NOAEL was derived, revealed only a reduction at the highest dose level tested making the data not suitable for dose-response modelling).	[127]
Sprague-Dawley rats.	Dams exposed to AA at 0, 25, 50 or 100 ppm (3.72 ± 0.28, 7.89 ± 1.70 and 14.56 ± 2.47 mg/kg bw/day, respectively) in drinking water (GD6 to PND 21), histopathological assessment performed at PND21. Exposure levels in offspring were examined by measurement of free AA and AA-Hb adducts on PND 14, and compared with maternal levels on PND 21. Another group of offspring received AA at 50 mg/kg (i.p.) directly three times a week from PND2 to 21.	Maternal neurotoxicity was evident at 100 ppm. No effect on the gestation period, number of implantations, live birth ratio and male pup ratio was observed. Offspring growth retardation (lowered body weights) observed. Offspring given AA i.p. exhibited obvious neurotoxicity. Free AA in serum and milk was detected in neither dams nor their offspring. The level of AA-Hb adducts in offspring was one tenth or less than that in dams. According to the authors, the internal level of AA in offspring exposed through maternal oral administration was insufficient to induce neurotoxicity due to limited lactational transfer.	NOAEL 100 ppm (no neurodevelopmental effects observed after maternal exposure). Maternal NOAEL 50 ppm.	[123]
Sprague-Dawley rats.	Dams exposed to AA at 0, 50, 100 or 200 ppm (9.9 ± 0.5, 16.7 ± 2.1 and 22.2 mg/kg bw/day, respectively) in the drinking water from GD10 to PND21. Histopathological assessment of offspring was performed at weaning and postnatal week 11. Neurotoxicity was quantitatively assessed with reference to nerve fibre density, percentages of degenerated and small caliber axons in the sciatic nerves, evaluation of synaptophysin immunoreactivity in cerebellum. Scoring of gait abnormalities were also performed.	Decreases of food and water consumption and suppression of body weight gain in the dams at ≥100 mg/L. Maternal neurotoxicity evident at 100 mg/l (abnormal gait, central chromatolysis of ganglion cells in the trigeminal nerves (already observed at 50 mg/L), dose-related increases of degenerated axons and myelinated nerves of < 3 μm in diameter, increase of synaptophysin-immunoreactive structures in cerebellar molecular layer), but at this dose level (according to the authors) no neurotoxicity was observed in offspring. Depression of body weight was observed from PND 2 through weaning from 50 mg/l in males and 100 mg/l in females. Decreased body weights of pups was dose-dependently observed from birth at the dose levels of > or = 50 ppm in males and > or = 100 ppm in females. Maternal malnutrition was apparent at ≥100 ppm during the lactation period. Therefore, according to the authors, maternal toxicity might account for the signs of AA-induced offspring toxicity	NOAEL 200 mg/l (however, signs of offspring neurotoxicity were observed at lower doses but authors report that this may be due to maternal toxicity and poor lactational transfer of AA) Maternal NOAEL 50 mg/l.	[124]

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Table 1 (*continued*)

Species	Dose and exposure	Neurotoxic effects	Neurodevelopmental NOAEL/LOAEL	References
		(e.g. increase of retained external granular cells in the cerebellum, increase in axonal density, and proportion of small myelinated nerve fibres in sciatic nerves at weaning).		
F344 rats.	Dams gavaged at 0, 0.1, 0.3, 1.0, 5.0 mg/kg bw/day (GD6 to PND85). On PNDs1–22, pups gavaged with the same dose as dams. At weaning (PND22), pups were pair-housed with a same-sex littermate and AA exposure continued at 0, 1, 3, 10 and 50 ppm in drinking water.	Decreased performance in an operant test of cognitive motivation at 5 mg AA/kg bw per day.	NOAEL 1 mg AA/kg bw/ day.	[126]
F344 rats.	0, 0.5, 1.0, 2.5, 5.0 or 10.0 mg/kg bw/day by gavage beginning on GD7, pups received daily gavage at the same dose as their dam from PND 1 through PND22.	No differences in righting reflex, forelimb hang, or open field measures of activity. Effects observed at the 10 mg/kg/day dose on negative geotaxis performance and a linear trend in fall-time latencies on rotarod performance on PNDs 21−22, with higher doses producing shorter latencies. The authors suggest that deficits in development and motor coordination are evident before weaning. No consistent dose-response on body weight gain in the male pups.	NOAEL 5 mg AA/kg bw/day. Maternal NOAEL 10 mg AA/kg bw/day.	[125]
Fischer 344 weanling rats.	Two-generation reproduction and dominant lethal study, 30/ sex and group, AA *via* drinking water (0, 0.5, 2.0 or 5.0 mg/kg bw/day for 10 weeks). Exposure of F0 females continued during gestation and lactation of F1 litters. F1 weanlings (30 per sex and group) were exposed for 11 weeks to the same dose levels and then mated to produce the F2 generation.	2.0 and 5.0 mg/kg bw/day resulted in systemic toxicity and increased head tilt and/or foot splay for rats in all dose groups. Implantations and live pups per litter reduced and survival for PND0 through PND4 reduced at the highest dose group. At the highest dose group peripheral nerves in the F1 exhibited axonal fragmentation and/ or swelling.	NOEL 2.0 mg/kg bw/ day NOEL for adult systemic toxicity including neurotoxicity $\leq$0.5 mg/kg bw per day.	[113]
Wistar rats.	Dams with litters (15/group) gavaged with AA at 0 or 25.0 mg/kg bw/day at PND 0−21. Male offspring were retained until PND91, with bw and grip strength evaluations.	Dosed dams exhibited progressive toxicity, including mortality (two), severely reduced feed and water consumption, b.w and b.w gain, and behavioural neurotoxicity (no sciatic nerve pathology). Nursing offspring at 25.0 mg/kg bw/day exhibited increased mortality and reduced bw associated with little/no milk in stomachs. Postwean males at 25.0 mg/kg b.w/day exhibited normal bw gain and increasing grip strength over time. Therefore, AA caused maternal toxicity; according to the authors the offspring effects during lactation were consistent with inanition from maternal toxicity. Postwean males exhibited recovery with no signs of AA-mediated toxicity. These results do not support the conclusions of Husain and colleagues [114] (similar study design).	NOAEL (neurotoxicity endpoints) 25 mg AA/kg bw/day. LOAEL in dams 25 mg AA/kg bw/day.	[119]
Sprague-Dawley rats.	AA orally from GD6 until day 10 of lactation (0, 5, 10, 15, or 20 mg/kg bw/day, 5 mL/kg bw, groups of 12 mated females each.	Increased pup mortality at 15 mg/kg/day group. Hindlimb splaying observed in dams of the two highest dosage groups. Pup body weight most sensitive indicator of developmental toxicity. Dose-related decreases in preweaning average weights were observed at all dose levels, although only transiently in the 5 mg/kg/day group. Average weight gain during the postweaning period decreased only in males at 15 mg/kg/day group. Significant decreases in average horizontal motor activity and auditory startle response in weanlings of the 15 mg/kg/ day group. Behavioural effect in F1 adult animals was a decrease in auditory startle response in females of the 15 mg/ kg/day group. There were no effects in the passive avoidance test or in the histological examination of the nervous system of preweaning pup or adult animals.	DNT NOAEL 10 mg/kg bw/day Developmental NOAEL $\leq$ 5 mg/kg bw/day. Maternal NOAEL was 5 mg/kg bw/day and LOAEL 10 mg/kg bw/day. Behavioural changes in the offspring were observed only at a dose which was also maternally toxic.	[104]
Wistar rats.	Protocol 1: 25 mg/kg bw/day administered orally to the mothers throughout the suckling period; animals were weaned at day 21. Protocol 2: normal rats aged 12, 15, 21 and 60 days were treated with 25 mg	Decreased levels of noradrenaline, dopamine, 5-hydroxytryptamine in pup brains at 4, 8 and 15 days of age (no changes observed in adult animals). Neurotransmitters were affected in a similar	NOAEL not determined.	[118]

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Table 1 (*continued*)

Species	Dose and exposure	Neurotoxic effects	Neurodevelopmental NOAEL/LOAEL	References
	AA/kg bw/day orally, for five consecutive days (saline as controls).	way in rat pups at 12–21 days of age (administered AA at 25 mg/kg per day), whereas these effects were not seen in rat pups that were 30 or 60 days of age at the initiation of dosing. Authors suggest that suckling and growing rats are more susceptible to AA neurotoxicity and that these effects were localized to regions which regulate motor activity and behaviour.		
Fischer 344 rats.	AA (20 mg/kg b.w/day) by gavage to time-pregnant rats from day 7 to day 16 of gestation.	AA did not affect the number, size, or bw of litters but did decrease the [^{3}H]spiroperidol binding in striatal membranes of 2-week-old pups. This effect could not be seen at 3 weeks of age. Scatchard analysis showed that AA changed the affinity as well as the number of dopamine receptor sites. No signs of maternal toxicity.	NOAEL not determined.	[120]

in reduced DA and 5-HT levels, while increased brain levels of noradrenaline, glutamate, aspartate and taurine [121].

Neurodevelopmental toxicity induced by AA has also been observed in brain tissue from chick embryos, which were exposed to a single dose AA at day 8,9 and 10 after fertilization and analysed at day 11 [122]. AA exposure starting at 0.1 mg/egg caused reduction in GSH and vitamin C levels in the hippocampal region, as well as induced mild structural damages at 0.3 and 0.4 mg treatment. Chick embryos exposed to 0.5 and 0.6 mg AA showed mild haemorrhages, necrotic damage and formation of vacuoles.

Contradictive results have been reported, where 100 ppm (approximately 15 mg/kg bw/day) AA exposure *via* drinking water in Sprague-Dawley dams during the same time span, starting at GD6 to PND21, did not induce neurotoxicity in the offspring examined through histopathological assessment on PND 21 [123]. AA levels in the offspring were determined by measuring free and AA-Hb adducts on PND14, where no free AA was found and the AA-Hb adducts were only one tenth or less than that in the dams, which could indicate limited lactational transfer. Another study using Sprague Dawley rat dams, given AA doses up to 200 ppm (approximately 22 mg/kg bw/day) from GD10 to PND21, also reported that no neurotoxicity could be observed in the offspring [124]. The authors reported signs of neurotoxicity during weaning in the pups, such as increased number of retained external granule cells in the cerebellum as well as increased axonal density and proportion of small myelinated nerve fibres in sciatic nerves. The authors speculate that this might be due to the observed maternal toxicity and could therefore not be directly correlated to AA.

4.4.2. Neurobehavioral alterations

Alteration in neurobehavior after AA exposure have been observed in rat pups exposed from GD7 until PND22, with negative geotaxis performance and a linear trend in fall-time latencies on rotarod performance in 10 mg/kg bw/day pups [125]. Pups exposed to 5 mg/kg bw/day through gestation and postnatal period showed decreased performance in an operant test of cognitive motivation [126] and AA dose of 5 mg/kg bw/day has been reported to decrease activity to 49 % at PND19−20 [127]. A cohesive study that assessed learning ability in rats at 1–8 month of age after exposure to 5.0 mg/kg bw/day starting *in utero* and throughout life span, showed a significantly lower performance in an incremental repeat acquisition (IRA) task compared to the control group [128]. AA-induced DNT has also been observed in zebrafish embryos, where AA exposure affected locomotor activity, measured as spontaneous tail coiling and hyperactivity [129].

In summary, the lowest NOAEL reported for neurodevelopmental toxicity in rats was 0.5 mg/kg bw/day after maternal exposure of AA in drinking water up to 3.0 mg/kg bw/day starting at GD6 until 2 years of age (Table 1, [110]). However, in most cases high exposure doses have been used and the neurodevelopmental NOAELs were below the dose levels of AA tested in the studies and therefore unknown. Notably, adult hippocampal neurogenesis, which is relevant also in the developing brain, is apparently affected at a dose level of 0.2 mg/kg bw/day. It is thus currently not possible to estimate a threshold dose for induction of neurodevelopmental toxicity in rodent studies for use in risk assessment.

4.5. Developmental neurotoxicity studied in in vitromodels

AA has been investigated as a DNT inducing agent using various cell models. AA exposure has been reported to attenuate vital processes during neuronal differentiation in several neuronal cell lines, with one common effect being reduced neurite outgrowth [99,130–133]. Impaired neurite outgrowth as well as sustained proliferation in retinoic acid-induced differentiating SH-SY5Y cells have been reported at AA concentrations starting as low as at 10 pM and 10 fM, respectively [130]. Chen and Chou also reported attenuated differentiation in the same cell line after AA exposure between 0.5 mM and 2 mM with down-regulation in the expression of neurofilament protein-L (NF-L), microtubule-associated protein 1b (MAP1b), MAP2c, and Janus kinase 1 (JAK1) [131].

Neuronal migration is an essential part of neurodevelopment. Ogawa et al. showed that AA altered the migration process, resulting in a distorted distribution of neurons [108]. AA starting at 0.35 mM has been reported to decrease the overall migration distance in human neural progenitor cells (hNPCs) grown as 3D neurospheres, but without specific alteration on neuron positioning [134]. AA exposure starting at 1 mM has also been reported to decrease the expression of neural cell adhesion molecule (NCAM) in SH-SY5Y cells [135]. NCAM plays an important role in neuronal development and synaptic plasticity. Downregulation of NCAM has also been reported after 0.5 mM AA exposure in the human neural stem/progenitor cells (NSPCs) U-1240 MG/F1B-GFP, which resulted in impaired neurosphere formation [136]. Furthermore, long-term exposure of AA has been implied to impair differentiation processes more severely than short-term exposure [137].

AA exposure alters the neuronal/glia cell ratio during differentiation. Attoff and collaborators reported a reduced number of neurons after AA exposure starting at 1 μM in the murine neural progenitor cell line C17.2 [130]. They further saw that AA exposure at 10 μM altered the ratio between different phenotypes in the cell cultures without affecting viability, which was confirmed by reduced expression of neuronal and astrocyte markers. AA exposure at 70 μM during 5 and 10 days of differentiation down-regulated genes involved in neural differentiation processes in the C17.2 cell line, such as neurogenesis (CHRDL1), axonal guidance (BMP4), neuronal connectivity (PLXDC2), axonogenesis (RTN4R), and astrocyte differentiation (S100B) [138]. AA exposure above 0.5 mM for 48 h has also been seen to impair neurogenesis by

inhibiting proliferation and induction of apoptosis, accompanied by increased ROS levels in C17.2 cells [103]. Increased ROS production after 0.5 mM AA treatment has further been reported in rat pheochromocytoma PC12 cells, where AA suppressed nerve growth factor (NGF)- or fibroblast growth factor 1 (FGF1)-induced proliferation in a time and dose-dependent manner [133]. This study also reported a reduction in the expression of the neural marker GAP43 and inhibition of PI3K-AKT-CREB signalling within 20 min after AA exposure. In contrast to the NGF stimulation, AA treatment in FGF1 stimulated PC12 cells showed reduced activation of ERK-STAT3 pathway, where the PI3K inhibitor (LY294002), but not the MEK inhibitor (U0126), was furthermore reported to synergize with AA [133]. Attoff and co-workers also showed that low concentrations of AA (1 and 70 μM) significantly affected the CREB activation pathway, reduced BDNF expression and attenuated retinoid acid induced signalling during differentiation of SH-SY5Y neurons [139]. The deregulated markers are all important for neuronal development.

AA exposure with concentrations between 0.1 mM and 2 mM were also reported to inhibit differentiation and proliferation in a time and dose-dependent manner in butyric acid induced differentiation of human glioblastoma U-1240 MG cells [131]. AA exposure during differentiation were reported to attenuate the expression of glial fibrillary acidic protein (GFAP), microtubule associated protein (MAP) 1b, MAP2c and janus kinase (JAK)1, and decreased the phosphorylation of extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK). These results indicate that butyric acid-induced astrogliogenesis was attenuated by AA and that this effect occurred concomitantly with down-regulation of MAPs expression and JAK-STAT signalling. Interestingly, AA exposure starting at 1 mM has also been seen to dose-dependently decrease DNA binding affinity of the Ikaros transcription factor, which is a transcriptional cell cycle repressor an effect that seemed related to an increased expression of casein kinase II (CK2) in SH-SY5Y cells [135].

The available *in vitro* studies indicate that AA impairs differentiation of neural progenitor cells and neuroblasts. Such effects may occur at low μM and sub μM AA concentrations. Altered expression and activation of several kinases and proteins like NCAM, CREB and BDNF that are important for neurogenesis and other neurodevelopmental processes are suggested to be involved in the AA induced developmental neurotoxicity. Most humans are exposed to AA on a daily basis through food, and the plasma level concentration of unbound AA has been estimated to be around 2 nM (peak plasma concentration) [55] in human exposed to 0.23 μg/kg/day. With an intake of 0.6–1.1 μg/kg bw/day [16,17], one can roughly assume unbound maximum plasma levels to be around 10 nM. For workers in AA industry, free AA plasma levels were reported to be 1.8 μM in average [69]. Assuming a placental transfer of AA of about 20 % [14] (giving a rough estimation of foetal plasma concentration of approximately 2 nM for the general population and a worst-case of 0.4 μM for workers), our conclusion is that the impaired neuronal differentiation *in vitro* at nominal concentrations in the sub μM range cannot be neglected (see Table 2 for more details on *in vitro* effects).

Putative modes of action of AA-induced DNT are summarized in Fig. 1.

4.6. Molecular interactions

In fully differentiated neurons, AA neurotoxicity is supposed to be associated with three modes of action suspected to induce neurite degeneration; inhibition of axonal transport [142–152], alteration of neurotransmitter levels and turnover [153–156], and direct inhibition of neurotransmission [157]. Based on *in vitro* and *in vivo* studies, AA acts on both central and peripheral neurons. It has also been reported that increased intracellular Ca^{2+} and calpains are involved in AA-induced neurite degeneration [158].

Several papers hypothesize that the primary site of action for AA neurotoxic effect is at the presynaptic part of nerve terminals and that the molecular initiating event of this process is the formation of AA adducts with specific sulfhydryl thiolate sites from proteins directly involved in the recycling of synaptic vesicles, thereby impairing the synaptic function [159]. Cysteine thiolate groups have clear regulatory functions in many critical neuronal processes [160], whereas protein valine, lysine, and histidine residues, which are also likely targets for GA, have unclear functional and toxicological relevance [88].

In isolated rat brain synaptosomes, one of several AA targeted proteins was the synaptosomal enzyme N-ethylmaleimide-sensitive factor (NSF), in which AA reacted with cysteines [161]. AA exposure to rat dopaminergic cells resulted in adduction of 100 proteins (≈0.7 % of the cell proteome), measured by shotgun proteomic LC–MS/MS analysis of peptides [162].

AA has been shown to react with certain cysteine residues in neuronal proteins such as Cys342 in the presynaptic Na^{+}-dependent DA transporter of rats [163]. Its position adjacent to polarizing amino acids in catalytic sites of proteins may lead to thiolate formation, and cysteine residues in so-called catalytic triads (three coordinated amino acids that can be found in the active site of some enzymes), that have been demonstrated to react preferentially with AA [164]. Thiolates in catalytic triads are typical targets for regulatory nitrosylation by endogenous nitric oxide, which can modulate synaptic transmission by reversibly inhibiting the function of proteins involved in the synaptic neurotransmitter vesicle cycle [160,165,166].

AA is a type-2 alkene and a soft electrophile that preferentially forms covalent bonds with soft nucleophiles such as cysteine [161,167]. The side chain nitrogen nucleophiles of histidine and lysine residues, as well as the protonated ε-amino group nitrogen of lysine, are harder moieties than the sulfhydryl thiolate, indicating that the sulfhydryl thiolate state of cysteine residues is the preferred target of AA (reviewed by [167]. However, nucleophilic reactivity is both a function of steric and electronic factors mediated primarily by protein tertiary structure [168].

Selenoproteins such as the GPx family play an important role in protection against oxidative stress (170). A difference between thiols and selenols is that the latter is dissociated at physiological pH and are therefore more reactive towards electrophiles. From a chemical point of view it is tempting to hypothesize selenolates as targets of acrylamide toxicity. However, we found no reports investigating AA- or GA reactivity towards selenols or selenolate. The GPx and Trx/Trx reductase system, which both use GSH as reductant, are the two main redox regulators of mammalian cells and the disruption of their activities can compromise cell viability [172], see Fig. 1. Functional Trx are important for neural development and protection [173], microtubule assembly [174], and is involved in the maintenance of mitochondrial homeostasis [175]. A summary of selenoproteins known to be involved in neurodevelopment, their subcellular locations and main sites of brain expression, can be found in the review by Pitts and co-workers [176].

In the CNS, thiol- and seleno-containing proteins involved in protection against oxidative stress are mainly located in mitochondria and in the cytoplasm of neurons [169–172]. Seleno-containing proteins include the thioredoxin (Trx) reductase family, involved in the regeneration of reduced Trx [173]. A difference between thiols and selenols is that the latter is dissociated at physiological pH and are therefore more reactive towards electrophiles. From a chemical point of view, it is tempting to hypothesize selenolates as targets of AA toxicity. However, we found no reports investigating AA or GA reactivity towards selenols or selenolates. The GPx and Trx/Trx reductase system, which both use GSH as reductant, are the two main redox regulators of mammalian cells and the disruption of their activities can compromise cell viability [174], see Fig. 1. Functional Trx are important for neural development and protection [175], microtubule assembly [176], and is involved in the maintenance of mitochondrial homeostasis [177]. A summary of selenoproteins known to be involved in neurodevelopment, their subcellular locations and main sites of brain expression, can be found in the review by Pitts and co-workers [178].

The rate of reaction of AA with high and low molecular weight-SH

Table 2
Studies summarizing AA developmental neurotoxicity *in vitro*.

Cell model	Toxic effects	Mechanisms investigated	Exposure levels leading to toxic effects	References
Human neural progenitor cells (hNPCs) grown as 3D neurospheres.	AA (0.0014, 0.0141, 0.0352, 0.0703, 0.1407, 0.3517, 0.7034 mM) decreased the overall migration distance (0.35 mM) without specific effects on neuron positioning, pointing toward a non-neuron-specific effect on migration. Also, total neurite length after AA exposure decreased (0.7 mM), and the number of neurons was significantly reduced at a subcytotoxic concentration of 0.35 mM.	Not investigated.	≥ 0.35 mM AA.	[134]
Mouse (KT98/F1B-GFP) and human (U-1240 MG/F1B-GFP) neural stem/progenitor cells (NSPCs)	0.5 mM AA inhibited neurosphere formation (definition of self-renewal ability in NSPCs) through the disruption of neurosphere architecture. Apoptosis was not observed in the AA-treated neurospheres. Impaired neurospheres formation correlated with a downregulation of cell-cell adhesion that was attributed to a decreased level of NCAM and reduced formation of NCAM/fibroblast growth factor receptor (FGFR) complex.	Mouse and human neurospheres: Reduced cell-cell adhesion associated with decreased NCAM expression and increased pERK. Decreased NCAM/FGFR complex after AA treatment.	Inbibited proliferation of NSPCs ≥1 mM of AA. Neurosphere formation reduced at ≥ 0.5 mM AA.	[136]
Murine neural progenitor cell line C17.2, neuroblastoma cell line SH-SY5Y.	AA reduced the number of viable cells by reducing proliferation and inducing cell death in undifferentiated cells in the neural progenitor cell line C17.2 and the neuroblastoma cell line SH-SY5Y. AA concentrations starting at 10 fM attenuated the differentiation process in SH-SY5Y cells by sustaining cell proliferation, neurite outgrowth was reduced at concentrations from 10 pM. AA reduced the number of neurons starting at 1 μM and altered the ratio between the different phenotypes in differentiating C17.2 cell cultures, and 10 μM of AA reduced the expression of neuronal and astrocyte markers.	Not investigated.	Low μM concentrations attenuated differentiation in both cell types.	[130]
Murine neural progenitor cell line C17.2.	Whole genome microarray analysis was performed after 5 and 10 days of differentiation. 30 genes were identified that are strongly associated with neural differentiation. Among the most highly upregulated genes were genes involved in neurogenesis (CHRDL1), axonal guidance (BMP4), neuronal connectivity (PLXDC2), axonogenesis (RTN4R) and astrocyte differentiation (S100B). The 30 markers of differentiation were further validated by exposure to among others AA and MeHg. AA significantly downregulated most of the selected markers after exposure to a non-cytotoxic concentration, indicating that AA might cause DNT. AA and MeHg resulted in structural alterations shown as reduced ratio of neurons in the cultures as well as reduced number of neurites per cell.	AA impaired neural differentiation (downregulation of genes involved in neurogenesis, axonal guidance, neuronal connectivity, axonogenesis and astrocyte differentiation).	70 μM of AA (IC10 was 70 μM ±21).	[138]
Neuroblastoma cell line SH-SY5Y.	AA resulted in decrease of NCAM expression. Moreover, AA induced the expression of CK2 protein and dose-dependently decreased the DNA binding affinity of the Ikaros transcription factor. Increased expression of CK2 appeared to mediate the effect of AA on NCAM and Ikaros.	AA decreased the Ikaros DNA binding activity *via* the CK2 pathway, resulting in a decrease of NCAM expression.	≥1 mM of AA.	[135]
Human neuroblastoma (SH-SY5Y) and human glioblastoma (U-1240 MG).	AA exposure inhibited cellular differentiation and cell proliferation in a time- and dose-dependent manner. Differentiation of SH-SY5Y and U-1240 MG cells were induced by retinoic acid (RA) and butyric acid (BA), respectively. AA co-treatment with RA attenuated SH-SY5Y expressions of neurofilament protein-L (NF-L), microtubule-associated protein 1b	Induced neurogenesis (SH-SY5Y) and astrogliogenesis were attenuated by AA. Effects were associated with down-regulation of MAP expression and JAK-STAT signalling.	≥0.1 mM of AA inhibited BA-induced extension of U-1240 MG cells. ≥0.5 mM of AA inhibited RA-induced outgrowths in SH-SY5y-cells.	[131]

(*continued on next page*)

Table 2 (*continued*)

Cell model	Toxic effects	Mechanisms investigated	Exposure levels leading to toxic effects	References
	(MAP1b), MAP2c, and Janus kinase1 (JAK1), while AA co-treatment with BA attenuated U-1240 MG expressions of GFAP, MAP1b, MAP2c, and JAK1, respectively. AA also decreased the phosphorylation of extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) in U-1240 MG cells.			
Murine neural progenitor cell line C17.2.	Effect of AA on neurogenesis in C17.2 neural progenitor cells was associated with significant inhibition of cell proliferation and induction of apoptosis and accompanied by increased ROS levels.	Inhibited proliferation, ROS formation.	≥50 μM AA (ROS formation) ≥2.5 mM of AA (viability and proliferation)	[103]
Differentiating Ntera2/clone D1 (NT2/D1) cell neurospheres.	Following induction of neuronal differentiation, AA reduced neurosphere viability but did not affect neuronal protein marker expression.	Not investigated.	Cell viability of AA: IC50 19.98×10^{-3}M±0.002 × 10^{-3} M.	[140]
Primary cultured neurons from Sprague Dawley rats.	AA exposure during the developmental stage delayed neuronal maturation, although without affecting cell viability.	Not investigated.	≥500 μM AA (mitochondrial dysfunction, reduced neurite length, βIII-tubulin, and synaptophysin) ≥50 μM AA increase in immature neuronal marker (DCX).	[99]
Rat embryo mid-brain micromass cultures.	The abilities of AA, 2,5-hexanedione and beta-aminopropionitrile to affect nerve cell differentiation were compared with their ability to affect cell survival over 5 days. AA inhibited neuronal differentiation by 50 % at a concentration (the IC50) of 15 μg/mL, and the corresponding IC50 for cell survival was 36 μg/mL.	Not investigated.	≥15 μg AA/mL (the IC50 of cell differentiation inhibition)	[141]
Neuronally differentiated mouse embryonal carcinoma P19 cells (P19 neurons), human neuroblastoma SH-SY5Y cells, rat adrenal pheochromocytoma PC12 cells.	Retinoic acid-treated P19 and SH-SY5Y cells and nerve growth factor-stimulated PC12 cells, allowed to differentiate for 6 days, were exposed for 48 h. AA also showed statistically significant toxicity in the P19 neurons, but not in the SH-SY5Y cells or the P12 cells.	Not investigated.	1 mM AA.	[137]
Rat pheochromocytoma PC12 cell line.	AA treatment suppressed nerve growth factor (NGF) or fibroblast growth factor 1 (FGF1)-induced PC12 cell proliferation in a time- and dose-dependent manner. 0.5 mM AA treatment resulted in significant decrease in differentiation of NGF- or FGF1-stimulated PC12 cells (quantification of neurite outgrowth), accompanied with the reduced expression of GAP-43, a neuronal marker. Together, the results revealed that NGF- or FGF1-stimulation of the neuronal differentiation of PC12 cells were attenuated by AA through the inhibition of PI3K-AKT-CREB signalling, along with the production of ROS	AA (0.5 mM) decreases the NGF-induced activation of AKT-CREB (0.5 mM). ERK-STAT3 activation induced by FGF1 was slightly reduced by 0.5 mM AA. They further showed that PI3K inhibitor (LY294002) synergized with AA (0.5 mM) and reduced cell viability and neurite outgrowth in NGF- or FGF1-stimulated PC12 cells. AA (0.5 mM) increased ROS activities in NGF- or FGF1-stimulated PC12 cells.	0.5 mM AA.	[133]
Neuroblastoma SH-SY5Y cell line.	AA alters neuronal differentiation at low concentrations by sustaining proliferation and suppress neurite outgrowth. AA interferes with genes in the RA receptor signalling pathway and CREB signalling pathway during differentiation at non-cytotoxic concentrations. Also, other markers important for neuronal differentiation, e.g. BDNF were affected at these concentrations and the same effect was seen at the protein level. AA also altered the protein expression of CREB and pCREB during differentiation.	AA alter expression of genes and proteins that are involved in normal neuronal differentiation and brain development such as BDNF and signalling *via* CREB and through the RA receptor.	1 and 70 μM during 9 days of RA-induced differentiation.	[139]

groups is slow, but can occur under physiological conditions [179,180]. It could be anticipated that some targets of the known thiol binding and neurodevelopmental toxicant methyl mercury and AA can overlap. This putative mode of action is suggested in AOP ID 17 "*Binding of electrophilic chemicals to SH (thiol)-group of proteins and/or to selenoproteins during brain development leads to impairment of learning and memory*" (https://aopwiki.org/). In AOP ID 17 (which is not yet endorsed by the OECD), a molecular initiating event is binding to selenoproteins leading to oxidative stress, cell injury/death, decreased neuronal network formation and function, and learning and memory impairment. AOP ID 17

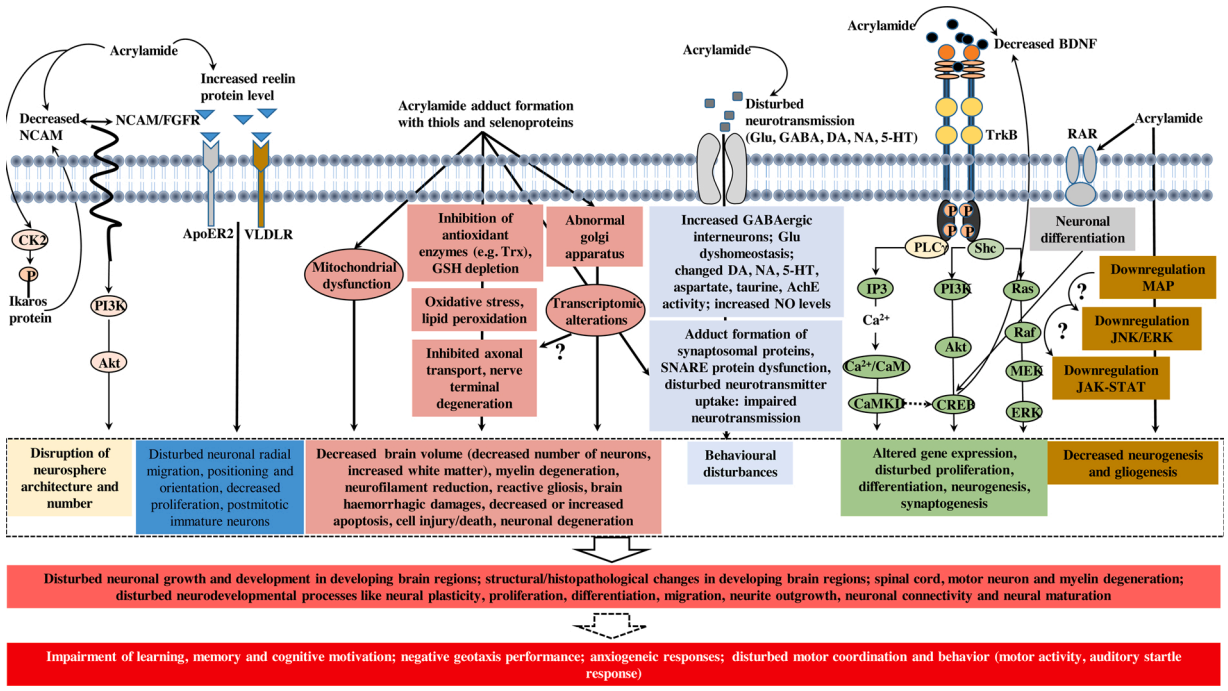

Fig. 1. Putative *in vitro* and *in vivo* mode of action for AA-induced DNT. The reported main effects of AA exposure are disruption of neurosphere architecture and number, disturbed neuronal migration processes, and neuronal proliferation. AA or GA may further interact with thiol/thiolate and seleno groups on proteins/enzymes central for neurodevelopmental function resulting in mitochondrial dysfunction, oxidative stress, transcriptomic alterations, inhibited axonal transport, and impaired neurotransmission. Interactions of AA with BDNF and CREB signalling and downregulation of the JNK/ERK and JAK-STAT pathways may lead to disturbed proliferation, neurogenesis and gliogenesis, differentiation and synaptogenesis. Alterations of these mechanisms and cellular processes necessary for normal brain development ultimately result in learning and memory impairment, anxiogenic responses, and disturbed motor coordination. More details of the *in vitro* and *in vivo* studies underlying the proposed mechanisms for AA-induced DNT can be found in Table 1 and 2. *Abbreviations: BDNF; brain derived neurotrophic factor, CaM; calmodulin, CaMKII; Ca2+/calmodulin-dependent protein kinase II, CK2; casein kinase 2, CREB; cAMP response element-binding protein, DA; dopamine, ERK; extracellular signal-regulated kinase, FGFR; fibroblast growth factor receptors, glu; glutamate, GSH; glutathione, IP3; inositol trisphosphate, JAK-STAT; janus kinase-signal transducers and activators of transcription, JNK; c-Jun N-terminal kinases, MAP; microtubule associated protein, MEK; mitogen-activated protein kinase, NCAM; neural cell adhesion molecule, NA; noradrenaline, NO; nitric oxide, PI3K; phosphatidylinositol 3-kinase, RAR; retinoic acid receptor, SNARE; soluble NSF attachment protein receptor, TrkB; tropomyosin receptor kinase B, 5-HT; 5-hydroxytryptamine (serotonin).*

is relevant for DNT following low dose and long-term exposure of chemicals like AA and for delayed adverse outcome.

In rat hepatocytes, GA depleted GSH content 1.5-times more readily than AA [181]. The stable or transitory interaction (binding) of *e.g.* methyl mercury with critical thiol and selenol groups in target enzymes can disrupt the activity of enzymes or the biochemical role of non-enzymatic brain proteins. Methyl mercury not only induces GSH depletion, but also binds to the thiol groups of Trx1 and the selenol group of TrxR and inhibit their function, a proposed molecular mechanism of mercury toxicity [182–184]. Some line of evidence support that AA may act in a similar way. In the study by Schwend and colleagues, Cys-74 in adenosine deaminase (in Jurkat cells) and Cys-73 in Trx (in Caco-2 cells) were found to be alkylated by AA [185]. Modification of Cys-73 is shown to affect the activity of Trx [186,187], and Trx is therefore a candidate mediating AA-induced cytotoxicity [185]. Sodium selenite and selenium dietary supplements significantly increased GSH and GPx levels and decreased MDA in the liver of AA exposed rats compared to rats that were exposed to AA only, suggesting selenite protection against biochemical changes in the liver of the rats which received high doses of AA [188]. In a recent zebrafish study, Trx and Trx-like proteins were significantly enriched among AA-modified peptides, where adduct formation was more evident in the mitochondrial Trx compared to the cytoplasmic Trx [189]. Here, it was suggested that the formation of AA-adducts with the active thiol groups of Trx could lead to their inactivation [189]. The catalytic reduction of H_2O_2 by peroxiredoxins and GPx involves the oxidation of catalytic thiol- and selenol groups on selenocysteine residues in GPx and cysteine in peroxiredoxins and Trx, and the recycling of these enzymes/cofactors is done by the thioredoxin reductase and GSH reductase systems, respectively [190]. This inactivation of the Trx system could prevent the regeneration of both methionine from its oxidized form [191], and the reduction to GSH [192]. Proposed modes of interference of AA with GSH and Trx antioxidant defence systems are shown in Fig. 2.

GA, the major metabolite of AA, is considered to be more reactive than AA. However, GA is, in contrast to AA, a harder electrophile that also forms adducts with hard nucleophiles such as nitrogen, carbon, and oxygen. Nucleotide residues of DNA contain abundant hard nucleophilic targets, consistent with the formation of GA adducts on adenine and guanine bases in animals [43,57,193] and the causality for genotoxicity and carcinogenicity of AA in rats. Nevertheless, GA can also form adducts with cysteinyl residues in thiol target proteins [194], but the role of GA in (developmental) neurotoxicity has been much less studied and is uncertain. It has been suggested that most genotoxic chemicals are hard electrophiles that form adducts with hard nucleophilic sites on nucleic acids (like GA reacting with nucleophilic centres on adenine and guanine of DNA [195]), while chemicals that produce non carcinogenic toxicity like DNT are often soft electrophiles (*e.g.* AA) that bind soft nucleophilic sites like cysteine sulfhydryl groups on proteins critical for neuronal function (reviewed in [168,196,197]).

In summary, several cellular processes and mechanisms taking place after AA exposure have been reported and are illustrated in Fig. 1. AA exposure of developing cells has been reported to disrupt neurosphere architecture, through decreasing cell–cell adhesion by reduced NCAM expression and formation of the NCAM/FGFR complex. This reduces the signal transduction of the PI3K/AKT pathway and the number of neurospheres [136] (for molecular mechanisms of NCAM function see

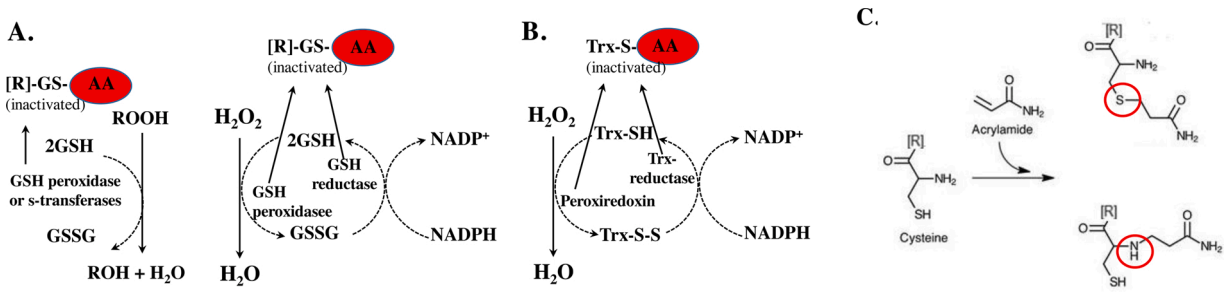

Fig. 2. Hypothesis of how AA can inactivate antioxidant enzymes or other enzymes critical for neuronal function by binding to the thiol/thiolate groups in their active sites leading to their inactivation ([R] denotes enzyme/protein moiety). A. Hydrogen peroxide (H_2O_2) from aerobic metabolism can be metabolized by GSH peroxidase in the cytosol and the mitochondria. GSSG is reduced back to GSH by GSH reductase at the expense of NADPH. Organic peroxides (ROOH) can be reduced either by GSH-s-transferases (GST) or GSH peroxidase. Accumulation of GSSG can occur under severe oxidative stress due to decreased capacity to reduce GSSG to GSH (illustration modified from [204]). AA can bind to thiolate groups on GSH reductase, peroxidases [114], and some GST isoforms leading to their inactivity. **B.** Graphical representation of the equilibrium between reduced thioredoxin (Trx-SH), oxidized Trx (Trx-S-S) and AA-inactivated Trx. Trx-reductase and peroxiredoxin catalyse the oxidation and reduction of Trx, respectively (illustration modified from [189]. AA can bind to the thiolate groups on Trx-reductase and peroxiredoxin and inactivate these enzymes. **C.** AA can form adducts with nucleophilic sites on amino acids of enzymes. Although soft nucleophilic groups like cysteine thiolate sites may be the most likely target for AA binding [159], it has also been proposed that cysteine may react with AA from both nucleophilic groups (–SH or NH_2) [205]. Nitrogen groups on lysine (ε-amino groups) and histidine (imidazole ring) residues are other examples of nucleophilic sites (moderate hard nucleophiles and less likely to react with AA). GA is a hard electrophile reacting readily with hard nucleophiles; however, it also forms adducts with cysteinyl residues in thiol target proteins. It is hypothesised that binding of AA or GA to enzymes may lead to impairment of their function, however, to what extent this may affect DNT (Fig. 1) is not known.

[198]), and activate CK2 and subsequently phosphorylation of the Ikaros protein leading to down-regulation of NCAM expression [135].

AA is further shown to disturb neuronal radial migration, positioning and orientation by increasing the number of reelin proteins in the hippocampus of rat offspring (that binds the receptors VLDLR and ApoER2 on the surface of radially-migrating neurons), and decrease proliferation leading to postmitotic immature neurons [114] (for review of reelin function see [199]).

AA is reported to induce GSH depletion or total thiol decrease [99,105,112,114–117,133], which may result in oxidative stress and/or elevated nitric oxide brain levels and further to lipid peroxidation. It may additionally disturb golgi apparatus, induce transcriptomic alterations [138,139], and inhibit axonal transport [142–152].

Structural changes reported after AA exposure are decreased volume of granular and molecular layer, increased volume of white matter, decrease myelinated nerves or myelin degeneration, reduce neurofilaments, and haemorrhagic damages are also shown [39,161,200,201]. Several authors furthermore describe mitochondrial dysfunction [99,105,115] and decreased or increased apoptosis [118,120,121].

AA forms adducts with proteins [103,107–109], which may lead to toxicity in nerve cell terminals and disturbed neurotransmission. In detail, AA exposure has been reported to alter DA levels or DA receptor binding [108,156], increase noradrenaline, glutamate, aspartate and taurine [121], increase GABAergic interneurons [112,114,138], decrease acetylcholinesterase activity [95,105,130], and decrease levels of noradrenaline, DA and 5-HT [118]. AA further inhibits the soluble SNARE protein SNAP-25 and N-ethylmaleimide sensitive factor by adduct formation [161,180,202]. This may ultimately lead to behavioural disturbances.

Decreased BDNF level [99,126,128,203] may lead to disturbed downstream pathways resulting in effects like impaired proliferation, differentiation, neurogenesis, and synaptogenesis. AA may disturb the PI3K-AKT-CREB signalling [133]. AA also decreases the phosphorylation of ERK and JNK reported to affect neurogenesis and astrogliogenesis [95,136]. Chen and Chpu (2015) report that butyric acid-induced neurogenesis and astrogliogenesis was attenuated by AA, and this effect occurred concomitantly with down-regulation of MAPs expression and JAK-STAT signalling [131].

Neuronal cell injury and death are reported in several studies [95,105,130], in addition to disturbed neurodevelopmental processes like neural plasticity, proliferation, differentiation, migration, neurite outgrowth, neuronal connectivity and neural maturation [111,127]. An overview of molecular mechanisms, pathways and factors involved in brain development is reported by Hessel and colleagues [74]. Compromising neurodevelopmental processes may ultimately lead to impaired learning, memory and cognitive motivation in animal offspring as shown in some studies [74], in addition to anxiogenic responses [111], negative geotaxis [125], motor activity and auditory startle response [104]. Other functional effects of AA exposure include disturbed activity in the open field test [111,127] and motor coordination deficits [125]. More details of the *in vitro* and *in vivo* studies underlying the proposed mechanisms for AA-induced DNT can be found in Table 1 and 2.

5. Knowledge gaps related to developmental neurotoxicity

From animal studies, it is documented that during critical periods of brain development, even low exposures to environmental toxicants, which rarely affect adults, can disrupt brain development [12,77]. This suggests that the nervous system of the developing foetus is particularly vulnerable to toxicant mediated interference. Data on AA show that the lowest NOAEL reported for neurodevelopmental toxicity in rats was 0.5 mg/kg bw/day [110]. However, in most cases the neurodevelopmental NOAEL in the studies listed in Table 1 is unknown, mostly due to the fact that animal experimental studies were not designed to elucidate thresholds of developmental neurotoxicity, but rather mechanistic aspects of toxicity. Furthermore, the *in vivo* endpoints studied are mostly histopathological observations. Based on the available animal studies, there is insufficient evidence to conclude, that the developing brain is more susceptible to AA toxicity than the adult brain. Notably, adult hippocampal neurogenesis that shares mechanisms occurring in the developing brain is affected by low doses of AA (0.2 mg/kg bw/day, [99]).

No studies on prenatal exposure to AA and neurodevelopmental functional effects in humans have been performed up to date. However, there are a few studies in human cohorts showing associations between prenatal dietary AA exposure *via* the maternal diet and reduced foetal growth including reduced head circumference. As the studies above clearly indicate that gestational exposure to AA may impair growth of the foetus, we conclude that there is an urgent need for further research to examine whether perinatal AA exposure might impair neurodevelopment and adversely affect neuronal function postnatally.

Although AA is rapidly metabolized and excreted, the AA and GA-Hb

adducts are more persistent and thus allows for good exposure estimates in pregnant women and in children. Hb adduct measurements should thus be included in future epidemiological studies of AA associated neurodevelopmental effects. Since none of the available PBTK models have included the foetus as compartment, more knowledge on the distribution of AA and GA into the foetal brain is crucial. Use of commercially available radioactive ^{14}C-AA, possibly also ^{14}C-GA (not commercially available but can be produced) in animal toxicokinetic experiments and in *in vitro* neuronal development studies, using IVIVE, can facilitate validation to such models.

Genetic polymorphisms in the AA metabolizing P-450 enzyme CYP2E1 have been identified in humans, resulting in differences in the Vmax of the enzyme [206]. Since both AA and GA have adverse effects, different catalytic activities of CYP2E1 or other factors that lead to high variability in the internal dose (*i.e.* differential metabolism to GA) may result in different spectra of adverse effects including the developing brain. Clearly, any assessment of health effects should consider the susceptibility of the biological activity of AA or GA at a target site. Both AA and GA are substrates for GSTs, however, it is not known which GST isoenzymes are involved [36]. Studies with purified and isolated GST enzymes are warranted to study the kinetics. Since polymorphism of GSTs are well described, including null-null (zero activity) for GST mu and theta in humans [36,207], the question rises how large the inter-individual variability may be with respect to GST polymorphism and susceptibility. This information can inform PBTK risk assessment models that relate to metabolic variability to internal dose and risk of neurodevelopmental effects.

A data gap exist regarding binding of AA and GA to thiol/thiolate and/or selenol/selenolate groups of proteins and enzymes critical for neurodevelopmental function. Resolving this is an important issue that will require more research, which could add more knowledge about the precise molecular sites of neurotoxicity that have not yet been clearly identified.

AA has been investigated as a DNT inducing agent using various cell models and has been reported to attenuate vital processes during neuronal differentiation [99,130–133]. Impaired neurite outgrowth in murine cell models as well as sustained proliferation in retinoic acid-induced differentiating cells have been reported at concentrations starting as low as at 10 pM and 10 fM, respectively [130], while some studies show effects at mM concentrations (e.g [131]). Depending on the cellular model, high acute *in vitro* concentrations may be required to generate intracellular cysteine adduct levels that exceed toxic thresholds. Therefore, long-term studies at relevant concentrations and in human neuronal stem cell (NSC) models are needed to mimic real-life exposures. Any robust approach relies on mechanistic models that are sufficiently representative of the human body and its organs/compartments. Human cell-based *in vitro* systems are recommended as the most relevant in the context of the 21st century approach to toxicity testing to reduce the uncertainty in extrapolation of results [208]. NSC models meet this recommendation since these consist of cell types (different types of neurons and glia including astrocytes) relevant for neurodevelopmental processes, are well characterized with respect to signalling, represent defined developmental time windows with relation to neurodevelopmental processes *in vivo* at the critical site of action, have some metabolic competence, and may represent human-relevant exposures [74,209–213].

In vitro studies should take into account metabolism and distribution of active species reaching intracellular targets, preferably by extensive *in vitro* kinetics experiments, enabling extrapolation from *in vitro* results to human tissue concentrations *in vivo* and back [208].

Very few studies have investigated potential neuroinflammatory processes in tissues of experimental animals as well as in appropriate primary cell cultures, as they are not present in NSC models. Microglia arise in the periphery, and their precursors migrate to the brain (reviewed in [214]).

There is a need to further investigate the role of AA as a stressor in AOP ID 17 "Binding of electrophilic chemicals to SH (thiol)-group of proteins and /or to selenoproteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory". To extend the mechanistic knowledge it should be elucidated whether AA can disturb neurodevelopmental processes like differentiation at long term, low level exposures in human relevant models, without affecting GSH redox state and ROS levels.

6. Future research recommendations

Based on knowledge gaps, we conclude that the most important research needs are to examine whether perinatal AA exposure might impair neurodevelopment and adversely affect neural function postnatally by;

(I) Assessing dose-response associations between AA exposure during pregnancy and neurodevelopmental endpoints in experimental models and humans;

(II) Investigating the importance of postnatal exposure to AA from breast milk on neurodevelopmental functional endpoints in humans;

(III) Establishing whether GST polymorphisms in human populations lead to differences in AA metabolism like formation of GA and metabolic detoxification leading to increased susceptibility of the unborn child;

(IV) Increasing current understanding of potential DNT of AA *versus* GA, in particular the primary molecular interactions, downstream cellular events (intracellular pathways involved are only partially understood) and sites of neurotoxicity need to be fully resolved;

(V) Examining vital neurodevelopmental processes after AA exposure by long term studies in human neuronal stem cell models to mimic real-life exposures;

(VI) Performing IVIVE studies relating the *in vitro* hazard characterization to the PBTK models obtained *in vivo* to estimate maternal oral doses and real-life risks;

(VII) Revealing potential neuroinflammatory processes in experimental models where functional microglia are present;

(VIII) Critically re-assessing the MOOE values based of peripheral neuropathy in adult rats [1,2] with full evaluation of epidemiological data and recent neurodevelopmental studies *in vitro* and *in vivo* to improve human risk assessment of AA exposure.

7. Literature search

A literature search was performed January 17th, 2020 on AA and/or GA and neurotoxicity/developmental neurotoxicity/neurological endpoints. A total of 2061 hits were obtained in this search after removal of duplicates. The list of publications was further reduced to 456 after filtering according to the exclusion criteria (*i.e.* substance identity not AA or GA monomers, publication not including AA or GA related neurotoxicity or neurological endpoints, review/discussion papers, conference abstracts). Of the 456 studies, 22 were classified as human studies, 375 as animal studies, and 59 as *in vitro* studies. Ten papers were added to the search; one human study [17] due to its perceived relevance although not formally addressing a neurotoxicological endpoint (head circumference) and 9 due to references found in the EFSA 2015 [1], Pennisi 2013 [215] and WHO 1985 [60] reports. A few additional papers published after January 2020 were also added to the search from Pubmed (*in vitro* and *in vivo* publications related to AA-induced developmental neurotoxicity, molecular interactions and modes of action of AA-induced neurotoxicity, neuro inflammation and hippocampal neurogenesis after AA exposure). Several of the 456 studies mentioned above were excluded. For the human studies, the focus of this review has been on the epidemiological and occupational studies of some size and thus only a few of the human case-studies were included. Of note, no studies directly addressing neurodevelopmental effects in humans were found in the literature search. Of the animal studies, those with exposure covering developmental periods and including endpoints addressing effects on the central nervous system or behavioural effects were

included. Similarly, for the *in vitro* studies only the models that were considered most relevant for addressing DNT were included in the review.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2021.02.006.

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