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Development of the Concept for Stem Cell-Based Developmental Neurotoxicity Evaluation

Ellen Fritsche,[*,†,1,2] Marta Barenys,[‡,2] Jördis Klose,[*,2] Stefan Masjosthusmann,[*,2] Laura Nimtz,[*,2] Martin Schmuck,[*,2] Saskia Wuttke,[*,2] and Julia Tigges[*,2]

[*]IUF - Leibniz Research Institute for Environmental Medicine, 40225 Düsseldorf, Germany; [†]Heinrich Heine University, 40225 Düsseldorf, Germany; and [‡]University of Barcelona, Spain

[1]To whom correspondence should be addressed. Fax: +49 (0) 211 3389 226; E-mail: ellen.fritsche@uni-duesseldorf.de.

[2]These authors contributed equally to this study.

ABSTRACT

Human brain development consists of a series of complex spatiotemporal processes that if disturbed by chemical exposure causes irreversible impairments of the nervous system. To evaluate a chemical disturbance in an alternative assay, the concept evolved that the complex procedure of brain development can be disassembled into several neurodevelopmental endpoints which can be represented by a combination of different alternative assays. In this review article, we provide a scientific rationale for the neurodevelopmental endpoints that are currently chosen to establish assays with human stem/and progenitor cells. Assays covering these major neurodevelopmental endpoints are thought to assemble as building blocks of a DNT testing battery.

Key words: stem cells; ESC; cellular and molecular biology.

For almost 20 years there has been considerable concern that chemical exposure might contribute to the increasing incidence of neurodevelopmental diseases in children (Bennett *et al.*, 2016; Grandjean and Landrigan, 2006, 2014; Schettler, 2001). Despite this concern, most chemicals have not been evaluated for their neurodevelopmental toxicity (Crofton *et al.*, 2012; Goldman and Koduru, 2000). The main reason for this data gap lies in the resource-intensity of the current guideline studies: EPA 870.6300 developmental neurotoxicity (DNT) guideline (U.S. EPA, 1998) and the draft OECD 426 guideline (OECD, 2007). These guidelines demand significant time, money and animals (Crofton *et al.*, 2012; Lein *et al.*, 2005) and are therefore not suited for testing large number of chemicals. The DNT TestSmart initiative originated and led by Alan Goldberg from the John's Hopkins University in 2006, took this issue up by bringing international scientists into communication on how to test for DNT with alternative methods (Lein *et al.*, 2007). Since then, international researchers have been developing concepts on how to use and interpret such alternative DNT methods with the final goal of regulatory application (Bal-Price *et al.*, 2012, 2015, 2018; Crofton *et al.*, 2011; Fritsche *et al.*, 2017, 2018; Lein *et al.*, 2005). The concept evolved that the complex procedure of brain development is disassembled into spatiotemporal neurodevelopmental processes that are necessary for forming a functional brain AND can be tested for adverse effects of compounds in *in vitro* assays (Bal-Price *et al.*, 2015, 2018; Fritsche, 2016; Lein *et al.*, 2007). Here, human-based systems are preferred because species differences in toxicokinetics, e.g., due to developmental timing, and/or toxicodynamics might affect responses to compounds (Dach *et al.*, 2017; Gassmann *et al.*, 2010; Gold *et al.*, 2005; Knight, 2007; Leist and Hartung, 2013; Masjosthusmann *et al.*, 2018; Seok *et al.*, 2013).

In the following paragraphs, we will provide the scientific rationales for the endpoints that are currently chosen for assay

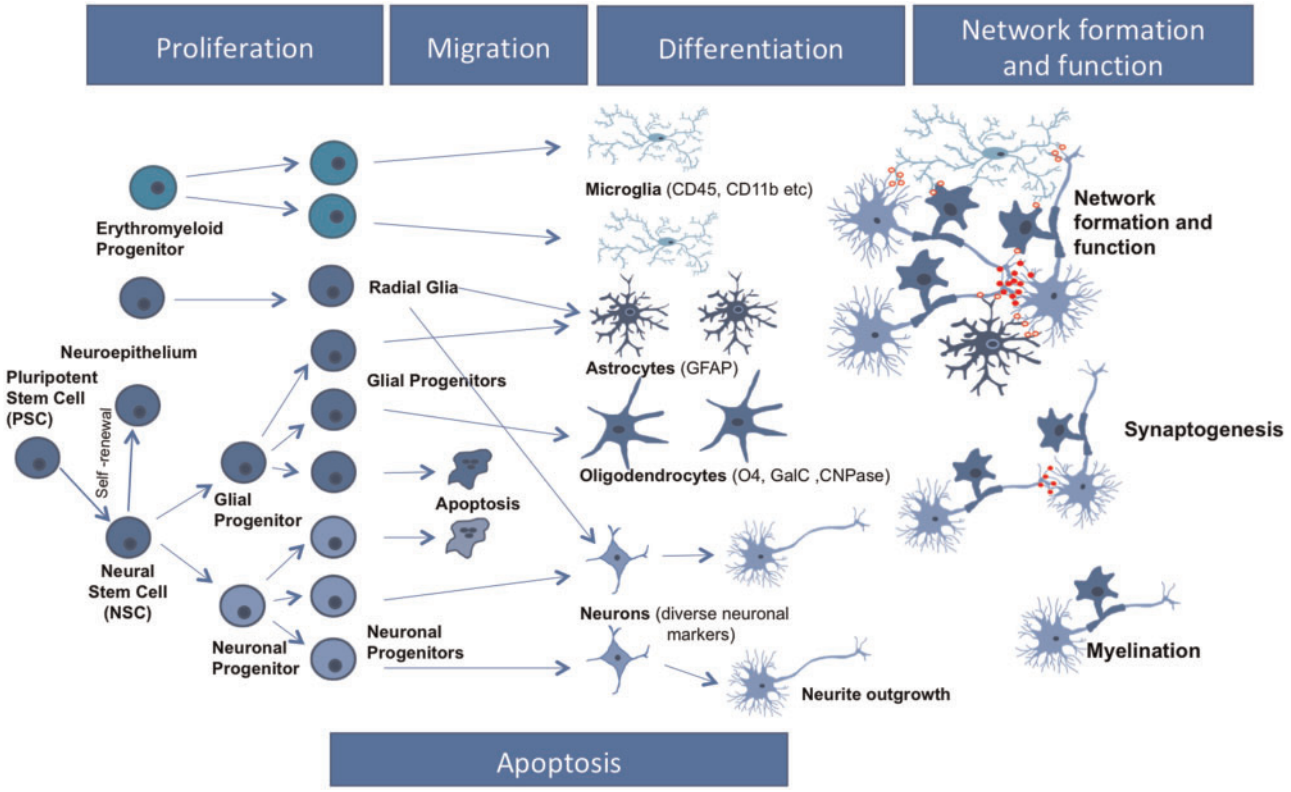

Figure 1. Neurodevelopmental processes essential for nervous system development. It is assumed that DNT toxicants exert their toxicity by disturbing at least one of these processes. Therefore, disturbances of the processes depicted here in blue boxes are key events of adverse outcome pathways relevant for DNT. From Bal-Price *et al.* (2018).

establishment with human stem/progenitor cells and depicted in Figure 1. Such assays are then thought to assemble as building blocks of a DNT testing battery covering neurodevelopmental endpoints over time.

ESC DIFFERENTIATION TO NEUROEPITHELIAL PRECURSORS/INDUCTION OF NEURONAL ROSETTES

During embryogenesis, stem cells develop into the primordium including the primordium of the central nervous system (CNS). During this neurulation, the neural plate and the neural groove form that lead to the emergence of the neural tube by fusing of the neural folds. Polarisation and patterning of the neural tube ultimately develop into the 3 major vesicles of the future brain: forebrain, midbrain, and hindbrain (reviewed by Silbereis *et al.*, 2016). Neural tube and axial defects of the vertebrate embryo belong to the most common developmental malformations in man. They include neural tube defects, which are among the most prominent birth defects in the human population with a prevalence of around 35 cases of spina bifida, 20 cases of anencephaly, and 10 cases of encephalocele per 100 000 births (CDC, http://www.cdc.gov/ncbddd/birthdefects/data.html; last accessed July 16, 2019). In addition, disruption of axial development might cause diverse craniofacial, limb, and cardiac malformations. During evaluation of developmental effects of chemicals and pharmaceuticals with experimental animal studies, neural tube and axial defects are frequently observed findings (Knudsen *et al.*, 2009). Examples for such human teratogens are anticonvulsants (e.g. valproate and carbamazepine), cytostatic agents (e.g. cyclophosphamide and methotrexate), and retinoids. An adverse outcome pathway framework was recently developed linking neural tube and axial defects to modulation of retinoic acid homeostasis (Tonk *et al.*, 2015). This framework was used as one of the building blocks for generation of developmental toxicity ontology (Baker *et al.*, 2018).

Human embryonic stem cells are able to differentiate into early neuroepithelial precursor (NEP) in the form of neural rosettes. These peculiar structures represent an *in vitro* primitive neural stem cell state with all the properties of neural plate cells and recapitulate the early neurulation events that bring the formation and closure of the neural tube (Elkabetz *et al.*, 2008; Lazzari *et al.*, 2006; Pankratz *et al.*, 2007). Such cells have a default anterior-dorsal pattern that is reverted by exposure to ventralizing signals such as sonic hedgehog and fibroblast growth factor 8 (Cowan *et al.*, 2004).

NEURAL PROGENITOR CELL PROLIFERATION

The brain is a highly organized structure and its development depends on the proliferation of a variety of progenitor cell types. When compared with lissencephalic species like mice and rats, brains of gyrencephalic species, like humans and ferrets, contain a larger variety of neural progenitor cells (NPCs) involved in e.g., the formation of the 6-layered neocortex. Also duration of proliferative NPC activity correlates with brain complexity, i.e. the phase of extensive progenitor self-renewal takes 2 weeks in mouse and 3 months in human developing brains. Such proliferative activity is directly coupled to the number of produced neural cells including neurons during corticogenesis, thus determining brain size, as well as to the formation of cortical gyri

and sulci. Here, centrosome-related proteins that determine spindle orientation and centrosome biosynthesis, the primary cilium, junctional adhesion molecules, and cell cycle length, determine NPC proliferation (comprehensively reviewed in Uzquiano *et al.*, 2018). A disturbance of NPC proliferation during brain development leads to significant alterations of brain morphology like a reduction of size, weight, or volume of the entire brain (microcephaly; de Groot *et al.*, 2005) or of individual brain structures (Moore *et al.*, 2006) having detrimental effects on the neurological outcome (Lang and Gershon, 2018; Ostergaard *et al.*, 2012). Besides genetic factors (Uzquiano *et al.*, 2018), also environmental elements can cause microcephaly. One recent example is Zika virus infection causing microcephaly in children (Devakumar *et al.*, 2018; Tang *et al.*, 2016).

NPC APOPTOSIS

Apoptosis is a crucial and strictly regulated event during brain development. Too much apoptosis can deplete the NPC pool in the developing brain. Here, loss of centrosome biogenesis in NPC, e.g., by deletion of *Cenpj* (*Sas4*), causes mitotic delay and an elimination of a subtype of NPC, apical radial glia (aRG), from the ventricular zone (VZ). This results in a thinning of upper cortical layers and microcephaly (reviewed in Uzquiano *et al.*, 2018). On the contrary, reduction in apoptosis due to inactivation of caspases or their copartners like *Apaf* can lead to morphological defects like hyperplastic brains. An apoptosis pathway involving caspases-3 and -9 is of particular importance in the developing brain. A reduction in apoptosis observed in Casp9$^{-/-}$ knockouts is thought to account for increased numbers of Bromodeoxyuridine-positive cells in the germinal zones of the brain that is consistent with an increased survival of NEP. As a consequence, both the VZ and forebrain cortical structures are expanded in these knockout mice, disrupting cortical organization and ultimately resulting in intracranial hemorrhage and death (Hakem *et al.*, 1998; Yoshida *et al.*, 1998). Apoptosis is therefore a well-balanced procedure where disturbance in either direction has detrimental outcomes.

RADIAL GLIA PROLIFERATION

Radial glia cells play key roles during cerebral cortex development. They are not a uniform cell type but are specified into different radial glia types with different functions across species. Describing the detailed functions of radial glia types extends beyond the scope of this article, for more in-depth information the interested reader is referred to excellent review articles (Gotz and Huttner, 2005; Uzquiano *et al.*, 2018). Briefly, aRG cells comprise the predominant neuronal progenitor cell type within the developing neocortex. They are highly polarized cells, exhibiting basal processes attached to the basement membrane, and apical processes linked by adhesion with cerebrospinal fluid in the ventricles. aRGs undergo asymmetric proliferative division into postmitotic neurons and neurogenic progenitors. In gyrencephalic species including humans, the initial pool of aRGs is greater than in lissencephalic species mainly contributing to neurogenesis through the production of a variety of basally located progenitors including basal radial glia-like cells (bRG), which are mainly intermediate progenitors (IPs). In primates including humans, IPs undergo several rounds of self-renewing before terminal differentiation. This higher neuronal production in gyrencephalic species impacts cortical size and folding (Borrell and Gotz, 2014; Fish *et al.*, 2008; Uzquiano *et al.*, 2018). Besides radial glia function as primary stem and progenitor cells that proliferate (see above) and give rise to neurons and glia, they also act as scaffolds for migrating neurons building the cerebral cortex architecture (Borrell and Gotz, 2014; Gotz and Huttner, 2005; Malatesta and Gotz, 2013). Due to the fundamental role of radial glia cells in brain development, disturbance of their biology will have detrimental results. For example, induced proliferation of the gyrencephalic ferret bRG leads to an expansion of the cortical surface area and the formation of new folds and fissures, while it increases surface area without creating new folds and fissures in the mouse (Nonaka-Kinoshita *et al.*, 2013). The instance that there are primate-human specific traits in brain ontogenesis that are targets of brain diseases and cortical malformations, like in the Miller-Dieker Syndrome, might explain why mouse models often fail to recapitulate patients' phenotypes (reviewed in Uzquiano *et al.*, 2018).

NEURAL CREST CELL/RADIAL GLIA/NEURONAL MIGRATION

Different neural cell types need proper migration during development. During embryogenesis, neural crest cells (NCC) migrate to distinct parts of the embryo developing into e.g. sensory and enteric neurons, Schwann cells, melanocytes, craniofacial structures like bone and cartilage, and chromaffine cells of the adrenal gland. Defective NCC migration and differentiation can cause a variety of diseases like cleft palate, hearing loss, Morbus Hirschsprung or CHARGE syndrome (Dupin and Sommer, 2012; Mayor and Theveneau, 2013).

Cortex development takes place during the fetal phase of brain development involving radial glia as well as neuronal migration (Borrell and Gotz, 2014). This neuronal migration process on scaffolds generated by radial glia migration is a fundamental neurodevelopmental key event, because radial glia as well as postmitotic differentiating cells migrate and differentiate over time into the main effector cells neurons, astrocytes and oligodendrocytes thereby ensuring normal brain structure and function (Carpenter *et al.*, 1999). Developmental brain disorders such as heterotopia and lissencephaly or diseases such as schizophrenia and epilepsy have been associated with disruptions of this cortical migratory process (Barkovich *et al.*, 2005; Bozzi *et al.*, 2012; Volk *et al.*, 2012).

ASTROCYTE DIFFERENTIATION/MATURATION

During the last decades, the view on astrocytes' physiology and their contribution to toxicity and disease has fundamentally changed. Although they were initially thought to play only a supporting and scaffolding role in brain, an increasing diversity of functions—also in a brain region-specific context—have now been appointed to this diverse cell type (reviewed in Hu *et al.*, 2016; Volterra and Meldolesi, 2005). Astrocytes create the brain environment, build up the microarchitecture of the brain parenchyma, maintain brain homeostasis, store and distribute energy substrates, control the development of neural cells, contribute to synaptogenesis, and synaptic maintenance, regulate cerebral blood flow, maintain the blood-brain barrier, and provide brain defense. Therefore, astroglia differentiation is a crucial event during brain development. Astrocytes express astroglial intermediate filament proteins like glial fibrillary acidic protein (GFAP) and vimentin, thus expression of GFAP is commonly used as a specific marker for astrocyte identification. However, it is to consider that *in situ* the levels of GFAP expression vary

quite considerably. For example, GFAP is expressed by virtually every Bergmann glial cell in the cerebellum whereas only about 15%–20% of astrocytes in the cortex of mature animals express GFAP. The same heterogeneity of astrocyte marker expression is also seen in different astrocyte *in vitro* methods (Lundin *et al.*, 2018). There is a large variety of different astrocytes present in brains probably conferring to the heterogeneity of astrocyte marker expression. For example, protoplasmic astrocytes are present in gray matter, while fibrous astrocytes are present in white matter yet with region-specific functions (Hu *et al.*, 2016). Another large class of astroglial cells are the radial glia, which are bipolar cells each with an ovoid cell body and elongated processes (described earlier Uzquiano *et al.*, 2018). After maturation, radial glia disappear from many brain regions and transform into stellate astrocytes (Adapted from Kettenmann and Verkhratsky, 2011).

Astrocytes seem to play a "yin-and-yang" role in health maintenance and disease of the brain. Their responses and roles in brain pathologies range from beneficial to adverse. Such astrocyte responses to a variety of stimuli are called reactive astrogliosis, a context-dependent progress undergoing a mild, moderate or severe substantial alteration of morphology and molecular function, i.e. releasing inflammatory factors. Reactive astrocyte responses might also be involved in the pathogenesis of neurodegenerative diseases like Parkinson's and Alzheimer's disease as well as amyothrophic lateral sclerosis. In addition, they seem to contribute to the pathogenesis of demyelinating diseases as well as brain aging (reviewed in Hu *et al.*, 2016). Despite just astrocyte marker expression, astrocyte function is crucial when studying astrocyte development. Four key astrocytic features of importance are (Lundin *et al.*, 2018): the uptake of the neurotransmitter glutamate, essential for synapse dynamics; inflammatory response to trauma; calcium signaling response to neurotransmitters; and the secretion of apolipoprotein E, a lipid and cholesterol transporter in the brain (Bazargani and Attwell, 2016; Khakh and Sofroniew, 2015; Yu *et al.*, 2014).

OLIGODENDROCYTE DIFFERENTIATION/ MATURATION

Oligodendrogenesis is necessary for proper brain function, as oligodendrocytes form and keep myelin sheaths around axons, a necessity for nerve cell function by enabling salutatory conduction. The peak of this process starts during the late fetal period and continues until the child's third year of age. Because myelin inhibits synaptogenesis and neuronal plasticity, this extended myelin production in humans prolongs the phase for learning capacities and memory (reviewed in Silbereis *et al.*, 2016). Several processes are involved in the generation of a sufficient number and proper functioning of oligodendrocytes. These include oligodendrocyte formation from oligodendrocyte progenitor cells (OPCs), maturation of OPCs, generation of myelin from matured oligodendrocytes and finally correct myelin sheet enclosure around axons. Disturbance of oligodendrocyte development may result in demyelination diseases that severely affect neuronal functioning and can be accompanied by impaired e.g. sensory, motor, or vegetative functions as well as memory (Baumann and Pham-Dinh, 2001; Nawaz *et al.*, 2015).

Oligodendrocyte development is linked to thyroid hormone (TH) action. Children suffering from the Allan-Herndon-Dudley Syndrome experience intrauterine brain hypothyroidism due to a mutation in the TH transporter monocarboxylate transporter 8 (MCT-8) resulting in delayed CNS myelination (Rodrigues *et al.*, 2014; Tonduti *et al.*, 2013). From the clinical data it is not clear if this myelination delay is due to less oligodendrocyte formation or maturation or a combination of both. However, *in vitro* studies using primary human neurospheres differentiating into oligodendrocytes suggest that TH induces oligodendrocyte maturation, but not formation, while in mouse neurospheres both endpoints are TH-dependent (Dach *et al.*, 2017). Thus, the oligodendrocyte maturation assay seems to be well suited to study TH disruption in developing human and mouse NPC.

NEUROGENESIS AND NEURONAL MATURATION

Neurogenesis in the human CNS begins shortly after the fusion of neural folds. Here, motor neurons in the ventral horn of the cervical spinal cord and neurons of certain cranial nerve nuclei in the brainstem appear first at gestational week 4 (Bayer and Altman, 2007; O'Rahilly and Muller, 2006). Prenatal neurogenesis continues throughout embryonic and fetal development mainly in the neocortex and the cerebellum. The cerebral cortex of a middle-aged male is estimated to be comprised of 16.34 billion neurons (Azevedo *et al.*, 2009), which are produced from neural stem or progenitor cells within the VZ and subventricular zone (SVZ) of the developing cerebral cortical wall. Of those, approximately 80% (13.07 billion) are estimated to be excitatory glutamatergic projection neurons (i.e. pyramidal and modified pyramidal neurons; DeFelipe *et al.*, 2002) and the rest are GABAergic inhibitory interneurons (reviewed by Silbereis *et al.*, 2016). Neurons are indispensable for life and their differentiation patterns are tightly regulated. Thus, modulation of neuronal differentiation into both directions (promotion or inhibition of neurogenesis) is considered as adverse. For example, reduced neurogenesis is thought to be involved in the pathogenesis of depressive mood disorders (Song and Wang, 2011) or the intellectual disabilities in Down Syndrome patients initiated during the fetal period (Guidi *et al.*, 2018; Stagni *et al.*, 2018).

Subsequent to neuronal differentiation, dendritic, and axonal (neurite) outgrowth followed by the formation of synapses are key cellular features associated with the functional maturation of the CNS. At midgestation, immature neocortical neurons have spread axons and instigated to expand dendrites that initiate an extended period of axon outgrowth, dendritic arborization and synaptogenesis extending into early childhood. Despite this general developmental concept, these processes vary substantially between brain layers, areas, and human neocortical neuronal subtypes (reviewed by Silbereis *et al.*, 2016).

NEURONAL SUBTYPE DIFFERENTIATION

During brain development neural stem and progenitor cells produce a variety of neuronal subtypes, which differentiate at different stages and in different regions of the brain. For example, glutamatergic neurons are generated from the VZ and SVZ of the dorsal mesencephalon (Fode *et al.*, 2000; Guillemot, 2007), GABAergic neurons from the ventral telencephalon (Casarosa *et al.*, 1999; Hansen *et al.*, 2010, 2013; Poitras *et al.*, 2007), while dopaminergic neurons are generated from several brain regions like the mesencephalon, hypothalamus, and retinal and olfactory bulbs (Alizadeh *et al.*, 2015; Arenas *et al.*, 2015; Zhang and van den Pol, 2015). Occurrence of neuronal subtype differentiation during early neurogenesis into 80% excitatory and 20% inhibitory neurons and their interactions are essential for the neuronal function of the CNS (DeFelipe *et al.*, 2002; Hansen *et al.*, 2010, 2013; Workman *et al.*, 2013).

SYNAPTOGENESIS/NEURONAL NETWORK FORMATION

During early neurogenesis neurons start to mature, connect, and become electrically active in the embryonic phase of neurodevelopment—between the fourth and fifth postconceptional week (Okado *et al.*, 1979; Zecevic and Antic, 1998). For the function of the CNS this neuronal maturation and the formation of synapses is crucial. The neuronal intercellular communication takes place at synaptic connections, which is associated with learning and memory through synaptic plasticity (Lundin *et al.*, 2018). When synaptogenesis is disrupted by a compound, neuronal network activity is altered (Robinette *et al.*, 2011). Here, synaptogenesis might converge on a variety of neurodevelopmental processes, i.e. key events, converging a compounds' effects on neuronal (subtype) differentiation, neurite outgrowth, axon or dendrite formation, dendritic spine development or synaptogenesis itself. Thus, this functional endpoint is a crucial readout for integration of neuronal function of the human brain.

CONCLUSION

Taken together, these neurodevelopmental processes have been identified somewhat as a minimum requirement for a proposed DNT testing battery, covering the complexity of neurodevelopmental processes as well as timing aspects of brain development. Moreover, they are considered for a DNT testing battery because there are stem/progenitor cell methods available allowing the set up of test methods for DNT evaluation (Bal-Price *et al.*, 2018). However, the current state of the science concerning identified endpoints is probably still at an early, immature state. The key events discussed here have clear rationales for their crucial function during brain development. Other key events like astrocyte differentiation and maturation, especially with regards to astrocyte heterogeneity, astroglia function, neuronal maturation, and neuronal network formation; however, are less well characterized. Moreover, the complexity of brain region-specific neural differentiation and function is not well understood, especially in the human context. Hormonal contributions to brain development are manifold and complex and chemicals with endocrine activities are suspected to interfere with neurodevelopment (WHO-UNEP, 2012). Here, interference with estrogen, androgen, retinoid, progesterone, peroxysome proliferator-activated receptor, or endocannabinoid signaling pathways might have implications for the developing brain at specific developmental stages. Especially sex hormone-related cellular and organ function is crucial for the development of gender-specific behavior, which follows species-specific traits (Wallen and Baum, 2002). As basic scientific knowledge on these neurodevelopmental key events deepens, implications for additional toxicity testing will arise and with this an evolvement of stem/progenitor cell-based methods for studying additional endpoints. However, the current state-of-knowledge is a satisfying start to put a DNT testing battery into action that covers neurodevelopmental endpoints across time.

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