Neural crest cell migration as a functional endpoint to test for developmental toxicity

Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.)

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Summary

Neurodevelopmental disorders affect 10-15% of children. For disorders like autism and attention deficit disorder, the prevalence has been rising, pointing towards environmental risk factors such as toxicants. At present, only few compounds are known as developmental neurotoxicants but most compounds have never been assessed for developmental neurotoxicity (DNT). To change this, novel high-throughput human cell-based assays are developed with the aim to model all critical steps of neurodevelopment. One step involves proper functioning of neural crest cells (NCC). NCCs are a particular fetal cell type that arises at the time of neurulation, delaminates from the neural tube and migrates to different places in the embryo to give rise to various cell types, including enteric and peripheral neurons, melanocytes and craniofacial skeleton. NCC function can be disturbed by toxicants such as retinoic acid.

The aim of the present thesis was to develop a high-throughput assay to measure human NCC migration and to investigate the molecular mechanisms that are disturbed by NCC migration-inhibiting compounds.

In a first step, an existing NCC migration assay was adapted for high-throughput. The new assay (cMINC) is experimenter-independent, suitable for automated image acquisition and reproducible. Proliferation-inhibition by toxicants was identified as potential confounding factor and further investigated. Using appropriate positive and negative controls, a preliminary prediction model was set up.

In a second step, performance of the assay was tested by screening a compound library consisting of compounds from several chemical classes (flame retardants, pesticides, drug-like compounds, polycyclic aromatic hydrocarbons, industrial chemicals), including known DNT compounds and negative controls. Of the tested compounds, 23/75 disturbed NCC migration at non-cytotoxic concentrations. Strikingly, many hits were halogenated or phosphorous organic compounds.

In a third subproject, mechanisms of migration-inhibition were investigated using polychlorinated biphenyls (PCBs) as an example. PCBs are a group of environmental contaminants composed of 209 congeners with varying chemical properties and biological activities. A structure-activity-relationship (SAR) was built for migration-inhibition and compared to literature data and own measurements. Subsequently, reduction of Connexin43 (Cx43) plaque number was identified as a potential key event in migration-inhibition.

In a follow-up project, testing of other NCC migration-inhibiting compounds revealed that several toxicants interfered with Cx43 localization, indicating that Cx43 mislocalization might be a general toxicity mechanism. Moreover, Cx43 is known to play an important role in NCC migration in vivo. All together, this leads to the conclusion that Cx43 mislocalization is a likely mechanistic step involved in migration-inhibition by a subset of NCC toxicants.

To conclude, the developed migration assay is suitable for medium to high throughput. Over 100 compounds have already been tested and > 30 NCC migration-inhibiting compounds identified. Some hits are shared with other assays, but there are also compounds unique to the cMINC assay. This indicates that the assay could give complementary information as part of a test battery to assess DNT. Future studies should address the role of Cx43 localization in human NCC migration in more detail.
Zusammenfassung


Das Ziel der vorliegenden Dissertation war das Entwickeln eines high-throughput Assays, um Migration humaner NCC zu messen und die daran beteiligten molekularen Mechanismen zu untersuchen.

In einem ersten Schritt wurde ein bestehender NCC Migrationsassay für high-throughput angepasst. Der neue Assay (cMINC) ist experimentator-unabhängig, reproduzierbar und kann automatisch gemessen werden. Proliferationshemmung durch Toxine wurde als mögliches Problem erkannt und angegangen. Mithilfe geeigneter Positiv- und Negativkontrollen wurde ein vorläufiges Vorhersagemodell erstellt.

In einem zweiten Schritt wurde der Assay angewendet um eine Substanzsammlung, bestehend aus Substanzen mehrerer chemischer Klassen, zu testen. Von allen getesteten Substanzen haben 23 von 75 NCC Migration bei nicht-toxischen Konzentrationen gehemmt. Auffallend viele dieser Substanzen waren halogenierte oder phosphorierte organische Verbindungen.

In einem dritten Teilprojekt wurden Mechanismen der Migrationshemmung am Beispiel von polychlorierten Biphenylen (PCBs) untersucht. PCBs sind eine Gruppe von Umweltschadstoffen mit verschiedensten chemischen und biologischen Eigenschaften. Eine structure-activity-relationship (SAR) wurde aufgestellt für Migrationshemmung und verglichen mit Literaturdaten und eigenen Messungen. Anschliessend wurde die Plaqueanzahl von Connexin43 (Cx43) als mögliches Schlüsselereignis identifiziert.

In einem Nachfolgeprojekt wurden weitere migrationshemmende Substanzen getestet. Mehrere dieser Substanzen reduzierten die Cx43 Plaqueanzahl, was darauf hindeutet, dass Cx43 Misslokalisierung ein allgemeiner Toxizitätsmechanismus sein könnte. Interessanterweise ist Cx43 auch wichtig für NCC Migration in vivo. Dies führt zum Schluss, dass Cx43 Misslokalisierung ein wahrscheinlicher mechanistischer Schritt ist, über welchen NCC Toxikantien Migration hemmen.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADD</td>
<td>attention deficit disorder</td>
</tr>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
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<tr>
<td>AOP</td>
<td>adverse outcome pathways</td>
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<tr>
<td>AP1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>AraC</td>
<td>cytosine arabinoside</td>
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<td>ASD</td>
<td>autism spectrum disorder</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>BMC</td>
<td>benchmark concentration</td>
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<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
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<tr>
<td>cMINC</td>
<td>circular MINC</td>
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<tr>
<td>Cx43</td>
<td>connexin43</td>
</tr>
<tr>
<td>Cx43pq</td>
<td>connexin43 plaques</td>
</tr>
<tr>
<td>CytoD</td>
<td>cytochalasin D</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNT</td>
<td>developmental neurotoxicity</td>
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<tr>
<td>DT</td>
<td>developmental toxicity</td>
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<tr>
<td>EC</td>
<td>effective concentration</td>
</tr>
<tr>
<td>EC75M</td>
<td>EC75 of migration</td>
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<tr>
<td>EC90V</td>
<td>EC90 of viability</td>
</tr>
<tr>
<td>EDNR</td>
<td>endothelin receptor</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethyl-2-deoxyuridine</td>
</tr>
<tr>
<td>EGCG</td>
<td>epigallocatechin gallate</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>EST</td>
<td>embryonic stem cell test</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FR</td>
<td>flame retardant</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell-line derived neurotrophic factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GJ</td>
<td>gap junction</td>
</tr>
<tr>
<td>GJIC</td>
<td>gap junction intercellular communication</td>
</tr>
<tr>
<td>GRIND2</td>
<td>grid-independent descriptors</td>
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<tr>
<td>GUI</td>
<td>graphical user interface</td>
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<tr>
<td>hESC</td>
<td>human embryonic stem cell</td>
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<tr>
<td>HNK-1</td>
<td>human natural killer-1 antigen</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>ITS</td>
<td>integrated testing strategy</td>
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<tr>
<td>KE</td>
<td>key event</td>
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<tr>
<td>logP</td>
<td>octanol-water distribution coefficient</td>
</tr>
<tr>
<td>MeHgCl</td>
<td>methylmercury (II) chloride</td>
</tr>
<tr>
<td>MIE</td>
<td>molecular initiation event</td>
</tr>
<tr>
<td>MINC</td>
<td>migration of neural crest cell</td>
</tr>
<tr>
<td>MTBE</td>
<td>methyl-, tert-butyl-ether</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NC</td>
<td>neural crest</td>
</tr>
<tr>
<td>NCC</td>
<td>neural crest cells</td>
</tr>
<tr>
<td>NHBA</td>
<td>number of hydrogen bond acceptors</td>
</tr>
<tr>
<td>NOAEL</td>
<td>no observed adverse effect level</td>
</tr>
<tr>
<td>NOAELV</td>
<td>no observed adverse effect level for viability</td>
</tr>
<tr>
<td>NRB</td>
<td>number of rotatable bonds</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
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<td>NTP</td>
<td>National Toxicology Program</td>
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<tr>
<td>p75</td>
<td>low affinity nerve growth factor receptor</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PBDE</td>
<td>polybrominated diethyl ethers</td>
</tr>
<tr>
<td>PBLs</td>
<td>peripheral blood-derived lymphocytes</td>
</tr>
<tr>
<td>PBPK</td>
<td>physiologically based pharmacokinetic</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyls</td>
</tr>
<tr>
<td>PM</td>
<td>prediction model</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PSC</td>
<td>pluripotent stem cells</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>QSAR</td>
<td>quantitative structure-activity relationship</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>superSHH</td>
<td>super sonic hedgehog</td>
</tr>
<tr>
<td>TB-BPA</td>
<td>Tetrabromobisphenol A</td>
</tr>
<tr>
<td>THR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>TPSA</td>
<td>polar surface</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>VPA</td>
<td>valproic acid</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Neurodevelopmental disorders and developmental neurotoxicity

1.1.1 Neurodevelopmental disorders

Developmental disorders

Developmental disorders are disorders that interfere with the normal development and growth of an infant up to adulthood. Developmental disorders can manifest in different ways, and include (1) fetal and neonatal death, (2) preterm delivery, (3) growth restriction, (4) structural alterations, and (5) functional alterations [Mattison 2010]. Major developmental defects (e.g. defects visible at birth) occur in approximately 3% of live births [Holmes & Westgate 2011]. Around 10% of these defects affect the nervous system [Dolk et al. 2010]. Examples include malformation of the brain (microcephaly, holoprosencephaly or hydrocephaly) and neural tube defects [Dolk et al. 2010; EPA 2013].

Functional alterations of the neurodevelopment are frequent

Whereas structural alterations are mostly visible at birth, functional alterations often only manifest later in life. These functional alterations range from delays in growth to deficits in neurological development and behaviour [NRC 2000]. Examples include attention deficit disorder (ADD), autism spectrum disorder (ASD), intellectual disability (also known as mental retardation) learning disability, cerebral palsy and impairments in vision and hearing [EPA 2013]. Such functional alterations are much more frequent than structural alterations. It is estimated that 10-15% of children are affected of such neurodevelopmental disorders [Bloom et al. 2010; Boyle et al. 2011; EPA 2013; Grandjean & Landrigan 2014]. These disorders can have severe consequences. They not only diminish the quality of life and academic achievement, they also have a socio-economic impact due to decreased lifetime earnings and increased health care [Gould 2009; Grandjean & Landrigan 2014].

Causes of neurodevelopmental disorders

Causes of neurodevelopmental disorders are not entirely understood. Most likely they result from a combination of genetic and environmental risk factors. Some disorders have a strong heritability (i.e. autism), whereas for others rather environmental risk factors are suspected [EPA 2013]. It is worrying that for some neurodevelopmental disorders, the prevalence has been rising in the last years. ASD rose from 0.1% in 1997 to 1% in 2010 and ADD rose from 6.3% to 9.5% of children in the same time range [EPA 2013]. This fast increase is not likely to come from genetic factors, but strongly points towards environmental risk factors.

1.1.2 Developmental neurotoxicity

Environmental risk factors include physical (hyperthermia, ultraviolet radiation, X-rays), biological (infections, nutritional deficiencies) and chemical (pharmaceuticals, drugs of abuse, pesticides, cosmetics, food additives) agents and conditions [NRC 2000]. If the neurodevelopmental disorder is caused by a chemical exposure of the mother during pregnancy or during lactation, this is termed ‘developmental neurotoxicity’ (DNT).
1 Introduction

Well-known developmental neurotoxicants
At present, there are only few compounds with convincing evidence to cause developmental neurotoxicity. They comprise a handful of drug-like compounds (NRC, 2000) and 11 environmental contaminants (Grandjean & Landrigan, 2014). The compounds, for which there is most longterm experience are polychlorinated biphenyls (PCB), lead and methylmercury (reviewed in Andersen et al., 2000). Other compounds or chemical classes include: toluene, arsenic, manganese, fluoride, organochlorine insecticides, polybrominated diethyl ethers (PBDE) and tetrachloroethylene (Grandjean & Landrigan, 2014). In the following, representative examples for drug-like and environmental compounds are given.

Drugs with known DNT potential
Several drugs are known to cause structural malformations of the nervous system. Retinoic acid (RA) derivates are vitamin A analogues and their teratogenic potential has been known for 30 years. Frequently, RA affects the central nervous system, but also malformations of the face, heart and thymus have been observed (Lammer et al., 1985).

Valproic acid (VPA) is used mainly as an antiepileptic drug. Prenatal exposure to VPA is associated with neural tube defects as well as craniofacial and limb malformations (Heyer & Meredith, 2017). However, drugs can also cause functional deficits. Prenatal exposure to VPA, thalidomide, misoprostol and ethanol has been linked to ASD (Arndt et al., 2005; Landrigan et al., 2012).

Lead: an environmental contaminant with known DNT potential
Similarly, there is evidence linking some environmental contaminants to functional deficits like ASD, ADD, dyslexia and IQ loss (Landrigan et al., 2012). One of the most well-known DNT compound is lead. Lead has been used for centuries for different purposes, in recent times as paint and petrol additive and in water pipes (Heyer & Meredith, 2017). Whereas acute lead poisoning often results in severe intellectual disability (Grandjean & Landrigan, 2006; Heyer & Meredith, 2017; Mendola et al., 2002), the subtle low level effects are much more worrying from a socio-economical perspective. Low level exposure has been associated with ADD symptoms, intellectual disability and violent crimes (Bellinger, 1994; Heyer & Meredith, 2017; Winneke, 2011). Many studies found that lead resulted in an IQ loss of 3-4 points (Yule, 1992). Whereas this likely has very little effect in people with average to high IQ, it will increase the number of people with intellectual disability (e.g. an IQ < 70) by a factor of three to four. This results in a tremendous socio-economical impact (Yule, 1992), as these individuals need more care and earn less, resulting in costs of 50 billions US$ per year in USA alone (Grandjean & Landrigan, 2014; Trasande & Liu, 2011).

Chemicals with limited evidence
For some other compound classes, there is limited evidence that they cause DNT based on prospective human studies. These comprise organophosphate pesticides, bisphenol A and other endocrine disruptors, perfluorinated compounds, polycyclic aromatic hydrocarbons (PAH) and automotive exhaust (Bennett et al., 2016; Landrigan et al., 2012). If data from animal experiments are included, there are roughly 100 compounds known to induce DNT (Bal-Price et al., 2012; Mundy et al., 2015).
1.2 Testing for developmental neurotoxicity

1.2.1 The need for DNT testing

Whereas the number of known and suspected DNT toxicants has been rising, there is still a vast amount of compounds that lack toxicity information. It is estimated that there are over 100'000 chemicals on the market, but comprehensive toxicity data is lacking for 90% of them (Crofton et al., 2012; Hartung & Leist, 2008). Even for marketed drugs, toxicity data is scarce: the teratogenic risk is only known for 10% (Adam et al., 2011; van Gelder et al., 2014). It is estimated that 2-3% (e.g. >2000) of compounds are reproductive toxicants (including DNT) (Hartung, 2009). To shed light onto the toxicity of existing chemicals, several initiatives have been started in Europe and U.S. For example, the European project REACH aims to perform toxicity testing for the 30’000 most frequent compounds, including reproductive toxicity testing for 5500 of them (EU, 2006; Hartung, 2009). However, to achieve this goal, a paradigm shift has to take place from animal-based towards high-throughput human cell-based testing for (developmental neuro-) toxicity.

1.2.2 Animal-based DNT testing

Conventional DNT testing is performed in rodents according to guidelines published by the U.S. EPA in 1998 (OPPTS 870.6300) and the OECD in 2007 (TG 426). For this purpose, animals (preferentially rats) are treated with the test substance during gestation and lactation to include all early periods of brain development. The offspring is then evaluated at different developmental stages for morphological, functional and behavioral effects. This includes motor activity, motor and sensory function, learning and behavioral tests and post mortem evaluation of brain weight and neuropathology (Bal-Price et al., 2015b; Makris et al., 2009; OECD, 2007). Testing is performed with at least three dose levels.

Limitations of animal-based DNT testing

DNT studies are very resource intensive. To have enough statistical power, approximately 1000 rat pups and 140 mates females are needed. Each animal has to be observed several times per week. Part of the pups are kept until 60 days after birth (PND60). Hence, it is estimated that such a study takes about three months and costs about €1 million (Crofton et al., 2012; Smirnova et al., 2014). For this reason, only about 100 compounds have been tested according to these guidelines so far (Makris et al., 2009).

Species-specificity limits reliability

Moreover, the usefulness of animal-based studies is questionable, due to low predictivity for humans. It is estimated that animal experiments have only a predictivity of 40-70% (Hartung, 2009; Olson et al., 2000). For example, the teratogenic dose of VPA to induce neural tube defects is 10 times higher in mouse and hamster compared to humans, whereas no defects are observed in rat, rabbit and monkeys (Nau, 1986). In other words: if VPA was tested only in rat (as suggested by the OECD guideline), the teratogenic potential would not have been discovered.

The causes of these tremendous species differences are not fully understood. It is generally assumed that they occur from differences in the pharmacokinetics (absorption, distribution, metabolism and elimination)
of the test compounds, as well as different distribution volumes, placental transfer rates and maternal plasma protein binding (Nau, 1986).

In the future, pharmaceuticals will often consist of ‘biologics’, e.g. peptides or proteins that have very species-specific effects, and hence animal experiments have only limited predictivity (Hartung, 2009).

1.2.3 Novel testing strategy

Based on the high number of untested chemicals combined with the low throughput and reliability of animal experiments, a novel testing strategy has been proposed by the National Research Council (NRC) with the aim to move from black box-like animal experiments to high-throughput human cell-based assays (Crofton et al., 2012; NRC, 2000, 2007). In this new strategy, different ‘disciplines’ like chemical characterization, toxicity testing, dose-response and extrapolation modelling, exposure data and risk contexts are combined (Fig. 1.1, left). The toxicity testing part consists of two tiers: In a first tier, toxicity pathways are elucidated in high-throughput manner whereas in a second tier, targeted testing of lower throughput is performed. The first tier is built of high-throughput biochemical, in vitro or in silico assays and non-mammalian model systems (Caenorhabditis elegans, Drosophila, zebrafish, ...). The targeted testing is for compounds that need more detailed information and consists of non-mammalian systems, mammalian models and other tailored assays (Fig. 1.1, right).

![Figure 1.1: Novel testing strategy suggested by the NRC.](image)

**Integrated testing strategy**

As the novel testing strategy relies on a battery of tests instead of only one assay, data analysis becomes more complicated. How should data of several assays be combined to a final result (high concern vs. low concern) if not all assays give the same result? This is the aim of the ‘integrated testing strategy’ (ITS). There are several different approaches possible (Fig. 1.2). In a weight of evidence approach, several tests are performed, but every test alone would not be enough to make a decision. However, all the data is integrated and together a statement can be made. In a similar way, a battery is a collection of assays that all must be performed to obtain a final result. This is different to a tiered strategy, where the
outcome of some tests may trigger different follow-up assays. In reality, combinations of the different approaches are also possible [Rovida 2014].

![Figure 1.2: Different approaches for an integrated testing strategy.](image)

In all scenarios, different assays (TEST) have to be conducted. Whereas in the 'weight of evidence' and 'test battery' approach all tests are executed, in a 'tired strategy' different tests are executed depending on previous results (Figure from Rovida, 2014).

**High-throughput assays**

One part of the testing strategy is based on high-throughput screening assays. These assays should shed light on the pathways of toxicity. The assumption is, that disturbing a pathway *in vitro* is predictive of effects *in vivo* ([Crofton et al., 2012; EPA, 2011]. Such pathways can be modeled in simple systems, e.g. high-throughput biochemical assays, *in vitro* cell-based assays or even *in silico* methods. These systems are then used in a first-tier screening to prioritize them for further *in vivo* or more detailed *in vitro* studies ([Bal-Price et al., 2012; Makris et al., 2009; NRC, 2007].

### 1.2.4 Existing in vitro assays for developmental (neuro)toxicity

**Assays for developmental toxicity testing**

In comparison to DNT testing, the field of (general) developmental toxicity (DT) testing is more advanced. Therefore, the DNT field can get inspiration from the DT field.

For some pathways of toxicity, high-throughput assays have already been setup. For example several assays have been developed that test for interference with nuclear receptors (i.e. estrogen receptor, androgen receptor, progestosterone receptor) (summarized in [Rotroff et al., 2013; van der Burg et al., 2015a]. In the frame of the endocrine disruptor program, >1000 compounds have already been screened (Judson et al., 2015; Lynch et al., 2017). On the whole organism level, a non-mammalian assay using zebrafish has been implemented and >1000 compounds have been screened for DT ([Padilla et al., 2012; Selderslaghs et al., 2010; Truong et al., 2014]. There is currently only one validated *in vitro* assay for DT. In the embryonic stem cell test (EST), three different readouts (cytotoxicity of mouse fibroblasts, mouse embryonic stem cells and differentiation of mouse embryonic stem cells) are combined in a test battery to predict the DT potential ([Seiler & Spielmann, 2011]. Subsequently, the EST has been combined with nuclear receptor assays and a zebrafish assay in the ReProTect and ChemScreen projects ([Kroese et al., 2015; Piersma et al., 2013; Schenk et al., 2010; van der Burg et al., 2015b]. However, at present, the throughput of the EST is still limited.

![Figure 1.2: Different approaches for an integrated testing strategy.](image)
A strategy for DNT testing
To replace animal-based toxicity testing, the idea is to assemble a test battery that contains assays for all important steps in neurodevelopment. This battery can be composed of simple, high-throughput signalling assays, similar (or identical) to the existing nuclear receptor assays for DT and more complex cell function assays. Such functional assays should cover a wide range of neurodevelopmental processes. Fritsche proposed that there are at least 22 cell functions that can or should be assessed for DNT (Fig. 1.3). (OECD, 2016).

Assays for DNT testing
For many of these 22 cell functions, test systems have been developed (reviewed in OECD, 2016). However, most of them are not yet enough characterized and not suitable for high throughput. The neurite outgrowth assay has a pioneering role. The assay consists of cultures of neurons that grow neurites. This neurite growth is disturbed by certain chemicals. Data acquisition takes place in an automated manner. Cells are live-stained and imaged using a high-throughput microscope. The neurite mass is estimated from the images using automated algorithms (Krug et al., 2013a; Stiegler et al., 2011). The assay can be automated and run at high throughput using 384-well format (Ryan et al., 2016; Sirenko et al., 2014). Importantly, the assay has successfully been setup in different laboratories and works with different cell types. Either human pluripotent stem cell-derived central neurons can be used (Harrill et al., 2011; Ryan et al., 2016; Sirenko et al., 2014), dopaminergic neurons (Krug et al., 2013a; Stiegler et al., 2011) or even peripheral neurons (Hoelting et al., 2016).

Another promising step was the assembly of a test battery in the framework of the ESNATS (Embryonic Stem cell-based Novel Alternative Testing Strategies) project. Different test systems model lineage induction of pluripotent stem cells, neuroectodermal induction, neuronal maturation, neurite outgrowth as well as neural tube formation and neural crest (NC) migration (reviewed in Krug et al., 2013b; Leist et al., 2013). A library of 30 compounds has been tested (Zimmer et al., 2014). However, at present, except for the neurite outgrowth assay, all of the ESNATS assays lack high throughput. From the existing assays, the NC migration assay has the potential to be optimised for high throughput.
1.3 The neural crest

The NC is a transient structure arising during early embryonic development at the time of neurulation and was first described by His (1868) in the developing chick embryo. Hoerstadius found out 1950 that cells of the NC contribute to the peripheral nervous system as well as to the facial skeleton. This is remarkable, because these two structures arise from different germ layers (ectoderm and mesoderm) and this violates the germ-layer theory. Therefore, in present time, the NC is often referred to as the 4th germ layer (Hall, 2000). The NC is unique to vertebrates and the cells build several structures specific to vertebrates, many of them related to predatory behaviour (Bronner & LeDouarin, 2012).

1.3.1 An overview on NC biology

Neural crest cells are stem-cell like

NC cells (NCC) are multipotent and have – albeit limited – capacity of self-renewal (Bronner & LeDouarin, 2012; Bronner-Fraser & Fraser, 1989, 1988). Therefore, NCCs can be considered as stem cells (Bronner & LeDouarin, 2012). However, as NCCs mature they become more restricted in their developmental potential and the term ‘progenitor cells’ is more appropriate (Crane & Trainor, 2006). Most NCCs differentiate during embryonic development. However, a subset of NCCs remains pluripotent until adulthood (Sieber-Blum et al., 2004).

Induction of the NC

During early neurulation, the neural plate forms and folds up to build the neural tube (Fig. 1.4). At the border of the neural tube and the epidermal ectoderm, the NC is induced (Hall, 1999; Le Douarin & Kalcheim, 1999). Extracellular signals like fibroblast growth factors (FGF), BMP and Wnts activate transcription of certain genes (‘neural crest specifiers’) that lead to NC induction (Milet & Monsoro-Burg2012).

These cells then undergo an epithelial-to-mesenchymal transition (EMT) that allows them to become migratory (Hall, 1999; Le Douarin & Kalcheim, 1999). Subsequently, these cells delaminate from the
neural tube, migrate in an organized manner to different places in the embryo and differentiate into a variety of cell types (Fig. 1.5) (Dupin et al., 2006; Le Douarin & Kalcheim, 1999; Le Douarin & Dupin, 2003).

**Figure 1.5:** Schematic representation of the important steps in the life of a NCC. Abbreviation: EMT: epithelial-to-mesenchymal transition.

**Migration and differentiation of NCC**

NCC migration is initiated spatiotemporally, e.g. in most species emigration starts once the neural tube is closed from the head and proceeds towards the tail (Bronner & LeDouarin, 2012).

NCCs can be classified into four ‘types’ along the rostro-caudal axis: cranial, vagal, trunk and sacral (Fig. 1.6). Depending from which region the NCCs emerge, they have different potential and differentiate into other derivates (Bronner & LeDouarin, 2012; Ruhrberg & Schwarz, 2010):

**Figure 1.6:** Different types of NCC differentiate into various cell types. NCCs can be classified into four types (left). Depending on the axial level, these NCC differentiate into other cell types (right). (Figure adapted from Simoes-Costa & Bronner, 2013).

1. Cranial NCC migrate to the pharyngeal (branchial) arches. Hereby they only migrate within even-numbered rhombomeres; odd-numbered rhombomeres remain NCC-free (Fig. 1.7A). Cranial NCC give later rise to parts of the craniofacial skeleton (jaw, inner and middle ear), connective tissue of the head, teeth, cells of the thyroid and parathyroid gland and cranial sensory ganglia (Douarin, 1982).

2. One part of vagal NCC are cardiac NCC that are involved in the outflow tract of the heart. The other part are enteric NCC that form later the enteric ganglia.
3. Trunk NCC can travel two pathways (Fig. 1.7B): Early migrating NCC take a ventromedial pathway in the rostral half of the somites and contribute to sensory and sympathetic ganglia and generate chromaffin and Schwann cells. Later emigrating NCC migrate dorsolaterally and generate melanocytes (Kuriyama & Mayor 2008).

4. Sacral NCC contribute also to the enteric nervous system. Additionally, NCC from all levels form melanocytes.

Figure 1.7: Migration routes of cranial and trunk NCC. (A) Cranial NCC migrate in odd rhombomeres to the pharyngeal arches. (B) Trunk NCC migrate on two different routes: ventromedial or dorsolateral. Abbreviations: OV: otic vesicle; NT: neural tube; NC: notochord; Ec: ectoderm; DM: dermomyotome; Scl: sclerotome. (Figures adapted from Nagoshi et al. 2009 and Clay & Halloran 2010).

NCCs can migrate in different manners: They often migrate in multicellular streams or even chains (Kulesa & McLennan 2015). For example cephalic NCC in Xenopus migrate initially as cell sheets, whereas in chick, NCC migrate in chains. In contrast to this, enteric NCC rather migrate as single cells (Theveneau & Mayor 2011).

NCCs have an enormous developmental potential. Their cell fate is determined by the axial level from which they arise and from the time of emigration. Grafting experiments showed that transplanted NCC from one axial level to another can adapt to the new environment, indicating that their cell fate is largely influenced by the environment (Baker et al. 1997 Bronner & LeDouarin 2012). However, NCC grafted at later time points rather maintain their initial fate, indicating that their potential gets restricted (Kuo & Erickson 2010; McKeown et al. 2003).
1.3.2 Molecular mechanisms of NCC migration

NCC migration is a complicated process. Depending on the axial level, NCC migrate at different time points and via different routes. To reach their target region, NCC have to migrate through other tissues/cell types. These cell types guide NCC by expressing permissive or repulsive signals (Fig. 1.7 and 1.8). During migration, NCCs should not disperse too much. Therefore, they form cell-cell interactions to stay together.

Interaction with the extracellular matrix

To move forward, NCC need to make interaction with components of the extracellular matrix. For this purpose, NCC express several integrin receptors that bind to fibronectin, laminin, vitronectin, collagen and tenascin (Delannet et al., 1994; McKeown et al., 2013). Integrins are linked to cytoskeletal proteins that in turn regulate NCC migration. Integrins are heterodimers composed of an α and a β subunit. NCC express multiple integrins, with some differences among species and NCC subtypes. Most NCC express α4, α5, αV, β1, β3 and β5, but also expression of α1, α3, α6, α8 has been reported (Haack & Hynes, 2001; Kil et al., 1998; 1996; McKeown et al., 2013; Pietri et al., 2003; Testaz et al., 1999). Depending on the type of integrin expressed, NCC are able to migrate on different substrates. Moreover, cranial NCC express syndecan4, a proteoglycan that binds to fibronectin (Clay & Halloran, 2010). Other signals include F-spondin, thrombospondin-1, chondroitin-sulfate proteoglycans and peanut agglutinin binding molecules (reviewed in Kuo & Erickson, 2010).
1.3 The neural crest

Molecular signals that promote grouping of NCC

It has been shown that if different cell types are mixed, they try to segregate from each other (Townes & Holtfreter, 1955; Xu et al., 2000). The reason might be, that cell-cell interactions between similar cells are stronger than interactions between different cell types (Steinberg, 1970; Xu et al., 2000). This is caused by expression of the same cell adhesion molecules or similar levels of a cell adhesion molecule (Xu et al., 2000). This mechanism might help pattern formation in the hindbrain (Martinez et al., 1992; Xu et al., 2000). NCC should stay in close contact, while they migrate through different, non-NCC tissue. NCC with different destinations were shown to express a different cadherin pattern (Nakagawa & Takeichi, 1995). Cadherins have homophilic binding specificity, e.g. affinity is high between cells that share the same cadherin isoforms and the same levels thereof (Inoue et al., 1997; Nakagawa & Takeichi, 1995).

In a similar way, gap junctional communication is more established within rhombomeres than across rhombomere boundaries (Martinez et al., 1992; Xu et al., 2000). The gap junctional proteins connexin also make stronger homophilic interactions than heterophilic ones (Johnstone et al., 2009).

Repulsive signals guide NCCs

Guidance of NCCs is also facilitated by repulsive signals. For example, restriction of cranial NCC to even-numbered rhombomeres and trunk NCC on particular paths is guided mainly by repulsive signals. NCCs express neuropilins and plexins, e.g. receptors for semaphorins. These molecules are secreted or membrane-bound proteins that are present in NC free regions (Kuriyama & Mayor, 2008). For example in the trunk region, semaphorin3F is expressed, which is repulsive for NCC and restricts them to the rostral half-somite (Kuriyama & Mayor, 2008). Similarly, semaphorin6 repels cardiac NCC (Kuo & Erickson, 2010).

In a similar way, ephrin ligands and Eph receptors guide NCC migration. Eph receptors are expressed in odd-numbered rhombomeres, whereas the ligands are expressed in the even-numbered rhombomeres (Trainor, 2003) to restrict NCC to the even-numbered rhombomeres. Eph receptors and ephrins are involved in both cranial and trunk NCC migration (Davy & Soriano, 2005). In the trunk, dorsolateral somites express ephrin ligands, that repel early migrating NCC and restrict them to the ventral path (Poliakov et al., 2004; Santiago & Erickson, 2002).

Another receptor system is Robo (expressed by trunk NCC) and the corresponding ligand Slit (expressed by gut mesenchyme) (Kuo & Erickson, 2010). Slit acts as a repellent for migrating trunk NCC in vitro and in vivo and keeps the trunk NCC on a ventral pathway (Kirby & Hutson, 2014; Kuriyama & Mayor, 2008).

Similarly, Erbb4 (expressed in rhombomeres 3 and 5) is involved in cranial NCC migration. If Erbb4 is ablated, NCC of rhombomere 4 ectopically migrate to rhombomere 3 (Trainor & Krumlauff, 2000). Although the above mentioned receptor systems act mainly repulsive, they can also act attractive in some situations. For example, semaphorin3c is attractive for cardiac NCC (Kirby & Hutson, 2014). Ephrins do act as positive signals for later emigrating trunk NCC that take the dorsolateral route (Poliakov et al., 2004; Santiago & Erickson, 2002). Moreover, Slit seems to be attractive for cranial NCC in rhombomere 4 (Kirby & Hutson, 2014).
Permissive and attractive signals

Besides repulsive signals, there are also receptor-ligand systems that provide permissive or attractive signals for NCC. For example, glial cell-line derived neurotrophic factor (GDNF) acts as a chemoattractant for enteric NCC, which express RET, the corresponding receptor \cite{Kuo2010,Young2011}. Similarly, SDF1 is a chemoattractant for cranial NCC. These NCC express the receptor CXCR4 and the ligand is expressed along their migration path \cite{Kuo2010}.

Another important receptor system are the endothelins. Whereas endothelin receptor (EDNR) A and the ligand endothelin (ET) 1 are important for cranial NCC, ENDRB and ET3 are involved in enteric NCC migration \cite{Pla2003}. Disruption of EDNRA/ET1 signaling results in malformation of the jaw, a structure derived from cranial NCC \cite{Pla2003}. Disruption of EDNRB/ET3 signaling leads to the absence of enteric ganglia and melanocytes, both structures that are derived from NCC \cite{Pla2003}.

Species differences in NCC migration

NCC differentiation and migration underlies strong species differences. For example, cadherin expression differs among species. Whereas mouse NCC express cadherin-6, chicken NCC express cadherin-7 and a particular form of cadherin-6, cadherin-6B \cite{Taneyhill2008}. And whereas avian NCC upregulate cadherin-7 upon emigration from the neural tube, Xenopus and mouse NCC upregulate cadherin-11.

Also the migration type displays differences: Cephalic NCC in Xenopus migrate rather sheet-like, whereas in the chicken, the cells migrate as streams or in chains \cite{Theveneau2012}. There are also differences in the genes involved in NC specification and early NC markers \cite{Gammill2003,Milet2012}.

More importantly, the timing and order of cranial NC EMT, delamination and migration varies between species. In the mouse and Xenopus embryo, NC delaminate at a time when the neural tube is still open, whereas in birds it happens at the time of neural tube closure. Moreover, delamination and EMT can take place at the same time (chicken) or in different orders (Xenopus and mouse) \cite{Theveneau2012}.

These species differences complicate the understanding of molecular mechanisms that drive NCC migration. Morover, because of these large species differences, it cannot be assumed that human NC biology is represented by any of these model organisms.

1.3.3 Neurocristopathies

If NCC function is disturbed, this results in disorders termed ‘neurocristopathies’. Because NCCs form various cell types in different parts of the body, the resulting syndroms look extremely heterogenous.

Some neurocristopathies affect only one organ or cell type (‘simple’), others occur at multiple sites and involve several cell types (‘complex’). Neurocristopathies can be classified in two types: Neoplastic and dysgenic \cite{Boland1997}. The characteristic of the first one is tumor formation and includes neuroblastoma, neurofibromatosis, schwannoma, pheochromocytoma (tumor of the chromaffin tissue of the adrenal medulla), medullary carcinoma of the thyroid and other endocrine tumors. The second type includes congenital malformations that result from disturbances in NC migration, colonization,
1.3 The neural crest

survival or differentiation. To the dysgenic neurocristopathies belong pigmentation disorders, craniofacial malformations like Waardenburg syndrome, DiGeorge syndrome and Treacher Collins syndrome, cleft palate or Hirschsprung’s disease. Two examples illustrate that different NC functions can be affected (Bolande, 1997).

Hirschspring’s disease: defective enteric NCC migration

Hirschspring’s disease affects 1 in 5,000 new-borns and is characterized by a part of the colon that lacks innervation and hence is not functional (Amiel et al., 2008). Patients suffer from constipation and the affected part has to be surgically removed. The entire enteric nervous system is formed by NC, on the one hand by vagal NC that migrate caudally through the gut, on the other hand by sacral NC migrating rostrally. Hirschspring’s disease occurs if vagal NCCs do not colonize part of the gut (Amiel et al., 2008). It is not entirely clear whether the NCC first migrate at normal speed and then abruptly slow down or if the enteric NCC migrate slower all the time (Farlie et al., 2004). During gut colonization, enteric NCC have to proliferate, migrate and later differentiate at the same time. If proliferation is disturbed, cell number will be reduced, which can also affect migratory behaviour or differentiation (McKeown et al., 2013). Several genetic mutations lead to Hirschspring’s disease and include - amongst others - RET, GDNF, ENDRB and ET3 (Amiel et al., 2008; McKeown et al., 2013).

Treacher-Collins syndrome: defective cranial NCC proliferation

Treacher-Collins syndrome affects 1 in 50,000 new-borns (Trainor, 2010). Patients have malformed mandibles, ears and teeth as well as cleft palate and eye problems (Trainor, 2010), clearly indicating the involvement of cranial NCCs. The disease is caused by mutations in the TCOF1 gene (Farlie et al., 2004). However, in Tcof1+/- mouse embryos, cranial NCC migration was not altered, but fewer migrating NCCs were observed (Dixon et al., 2006). Thus, it seems that Treacher-Collins syndrome is the result of reduced proliferation capacity or survival of NCC (Dixon et al., 2006; Trainor, 2010).

1.3.4 Toxicants disturbing NCC function

Besides genetic causes, neurocristopathies can be caused by toxicants.

Ethanol and RA are well-known NC toxicants

The most well-known NC toxicants are ethanol and RA (Bolande, 1997). In particular RA leads, amongst other symptoms, to cleft palate and malformation of craniofacial structures in new-borns if administrated to pregnant women (Bolande, 1997; Lammer et al., 1985). Several in vivo and ex vivo studies with different species (avian and rats) confirmed that indeed NCC migration was disturbed by teratogenic RA concentrations, particularly cranial NCC migration (Menegola et al., 2004; Shankar et al., 1994; Smith Orris et al., 1999; Smith-Thomas et al., 1987; Usami et al., 2014). Interestingly, albeit NCC migration was inhibited, migration of somite cells was not affected by RA (Smith Orris et al., 1999), indicating a specific effect of RA on NCC. Similarly, animal experiments confirmed that ethanol interferes with migration and patterning of (cranial) NCCs in mice and Xenopus (Shi et al., 2014; Usami et al., 2015; Van Maele-Fabry et al., 1995).
Triazole-derived fungicides alter cranial NCC migration
Over the last years, evidence accumulated that triazole-derived fungicides are also toxic to NCC. Some case reports indicate craniofacial alterations in new-borns of mothers treated with high concentrations of fluconazole (Menegola et al., 2005; Pursley et al., 1996; Sanchez & Moya, 1998). Rat in vivo and whole embryo culture studies confirmed that triazole-derived fungicides altered migration of cranial NCC streams (Di Renzo et al., 2007; Menegola et al., 2003). As a mechanism, interference with RA metabolism has been suggested (Menegola et al., 2004; Papis et al., 2007).

Evidence for cyclopamine and VPA as NC toxicants
Cyclopamine is well-known for its teratogenic effect to induce cyclopia (Binns et al., 1963). However, cyclopamine also induces holoprosencephaly and craniofacial defects, likely via a mechanism involving cranial NCC (Coventry et al., 1998; Dunn et al., 1995). A study performed with Xenopus embryos found that cyclopamine specifically induced cell death in cranial NCC but not in trunk NCC or in other cell types. Even if treated cranial NCC were grafted into untreated host embryos, the NCC did not recover, indicating that the compound acted directly on the NCC and not (only) on the surrounding tissue (Dunn et al., 1995).

VPA, a well-known DNT compound, was found to inhibit attachment of chicken neural tube explants and reduce the amount of NCC migrating out of the explant (Fuller et al., 2002).

1.3.5 NCC function assays
In vivo experiments and whole embryo cultures
Up to now, most research about NC toxicants was performed using animal experiments. One way to study NC biology are in vivo experiments. In this case, the pregnant animal is treated at the time of neural tube closure and effects on the embryo or the new-born are studied at a later time point (i.e. Di Renzo et al., 2007). Another possibility are ‘whole embryo cultures’. For Xenopus, this means treatment of fertilized eggs in vitro (Papis et al., 2007). For studies with rats, embryos have to be explanted. In that case, embryos are explanted at around the time of neurulation and treated in vitro with the compound of interest (Menegola et al., 2004, 2005, 2000). The advantage of in vivo experiments is, that effects on the whole organism are visible. However, except for Xenopus, it is not possible to see the single NCC migrating.

Neural tube explants as a common ex vivo method
This disadvantage is circumvented in the case of neural tube explants, the most common method used to study NC migration. Embryos are explanted from the pregnant mother and the neural tube is removed from the embryos and cultured in dishes. After 1-2 days, NCC migrate out of the explant and their migration can be followed. This technique has been used to study NC migration of several species (Xenopus, chicken, mouse and rat) and depending on the part of neural tube that is excised cranial or trunk NC can be obtained (Fuller et al., 2002, Kulesa & Fraser, 1998, Milet & Monsoro-Burq, 2014, Pfaltzgraff et al., 2012, Usami et al., 2014, 2015, Walheim et al., 2012). Using an adaptation of the method, also enteric NCCs can be obtained (Bergeron et al., 2013). The advantage of neural tube
The neural crest explants is that they are relatively easy to obtain (no difficult cell culturing involved), but the disadvantage is that the explantation is quite time-consuming and laborious and hence only few explants can be obtained at one time, which makes the system not suitable for high throughput. Hence, most studies up to now investigated only one compound at a time. Only one study used this approach to test 13 compounds for their effect on rat cranial NCC migration (Usami et al., 2015).

**Human stem cell-derived NCCs as a novel alternative**

With the development of human embryonic stem cell (hESC) technology, human NCC became available. In the last years, several differentiation protocols have been developed that allow differentiation of human pluripotent stem cells (PSC) towards NCC (Chambers et al., 2012; Lee et al., 2010; Liu et al., 2012; Mica et al., 2013; Reinhardt et al., 2013). This strategy has not only the advantage that for the first time human NCC can be studied *in vitro*, but also that large cell batches can be generated, making the system potentially suitable for high throughput.

Following this strategy, Zimmer et al. (2012) setup a wound healing assay with human NCCs. In this assay, NCCs are seeded into 48-well plates and after two days, a scratch is introduced using a pipette tip (Fig. 1.9). This results in a cell-free area that is then repopulated by migrating NCC within 48h. During this migration phase, toxicants are added. At the end of the assay, cell nuclei are stained with H-33342 and images of the scratch region are acquired manually to evaluate the amount of migrated cells. Using this strategy, approximately 50 compounds have been successfully tested. Among the positive hits (e.g. migration-inhibiting compounds) were known NC toxicants (VPA, several triazol-derivates), DNT compounds (methylmercury chloride, As$_2$O$_3$, CdCl$_2$, lead acetate), some drugs (gleevec, geldanamycin, interferon $\beta$) and persistant organic pollutants (PCB, PBDE) (Dreser et al., 2015; Zimmer et al., 2012, 2014).

![Figure 1.9: Overview of the MINC assay.](image) Cells are seeded, two days later a scratch is introduced and toxicants are applied. After 48h, migrated cells are counted using H-33342 staining (Figure modified from Dreser et al., 2015).
1.4 Aims of this thesis

Toxicants contribute to the increased prevalence of neurodevelopmental disorders. However, only few toxicants have been studied for their DNT potential due to expensive animal testing. Therefore, the testing strategy is changing towards human cell-based in vitro assays to screen compounds in a high-throughput manner.

One group of (neuro)developmental disorders is caused by defects of NC function. Such neurocristopathies can be caused by toxicants, but at present there are no high-throughput human cell-based assays available. Only one human cell-based NC assay has been available at the onset of this thesis. However, it was not suitable for high-throughput measurements. Therefore, a goal of the present thesis has been to develop a high-throughput assay to measure NCC migration and to investigate the molecular mechanisms that are disturbed by NCC migration-inhibiting compounds.

The objectives of this study can be detailed as follows:

1. Development of an in vitro NCC migration assay using hESC-derived NCC. The assay should be experimenter-independent and suitable for high-throughput testing. Appropriate positive and negative control compounds were to be used to calibrate the assay and to setup a prediction model.

2. Testing the suitability of this assay for screening purposes by screening a proof-of-concept chemical library for compounds interfering with NCC migration.

3. Investigation of a mechanism of toxicity relevant to NCC migration-inhibition by environmental chemicals. For this purpose, the toxicant class of polychlorinated biphenyls (PCBs) has been selected and studied in detail.

4. Investigation whether the mechanism identified for PCBs is a common pathway of toxicity shared by other NCC migration-inhibiting toxicants.
2 Results. Manuscript 1

Design of a High-Throughput Human Neural Crest Cell Migration Assay to Indicate Potential Developmental Toxicants

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Summary
Migration of neural crest cells (NCCs) is one of the pivotal processes of human fetal development. Malformations arise if NCC migration and differentiation are impaired genetically or by toxicants. In the currently available test systems for migration inhibition of NCC (MINC), the manual generation of a cell-free space results in extreme operator dependencies, and limits throughput. Here a new test format was established. The assay avoids scratching by plating cells around a commercially available circular stopper. Removal of the stopper barrier after cell attachment initiates migration. This microwell-based circular migration zone NCC function assay [cMINC] was further optimized for toxicological testing of human pluripotent stem cell (hPSC)-derived NCCs. The challenge of obtaining data on viability and migration by automated image processing was addressed by developing a freeware. Data on cell proliferation were obtained by labelling replicating cells, and by careful assessment of cell viability for each experimental sample. The role of cell proliferation as an experimental confounder was tested experimentally by performing the cMINC in the presence of the proliferation-inhibiting drug cytosine arabinoside (AraC), and by a careful evaluation of mitotic events over time. Data from these studies led to an adaptation of the test protocol, so that toxicant exposure was limited to 24 h. Under these conditions, a prediction model was developed that allows classification of toxicants as either inactive, leading to unspecified cytotoxicity, or specifically inhibiting NC migration at non-cytotoxic concentrations.

Keywords: cell tracking, cell proliferation, high content imaging, developmental toxicity, human stem cells

1 Introduction

The design and optimization of human-relevant test systems for developmental toxicity is an important challenge for modern toxicology research. Animal-based tests lack the throughput required to evaluate the large number (Rovida and Hartung, 2009; Crofton et al., 2012; Smirnova et al., 2014; Bennett et al., 2016) of untested substances, and they are animal- and resource-intensive. Moreover, they require that results are extrapolated from the test animal to humans, a particularly difficult task where the development of the immune and nervous systems are concerned. Thus, it has been proposed to focus on tests based mainly on human cells. A recent study based on 10,000 chemical profiles modelled in the Tox21 effort demonstrated the advantage of human cells over animal cells in predicting human toxicity hazard (Huang et al., 2016). The increased use

Abbreviations
AraC, cytosine arabinoside; BDNF, brain-derived neurotrophic factor; BMC, benchmark concentration; cMINC, circular MINC; DMSO, dimethyl sulfoxide; DNT, developmental neurotoxicity; EC, effective concentration; EdU, 5-ethynyl-2’-deoxyuridine; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; GUI, graphical user interface; hESC, human embryonic stem cell; HNK-1, human natural killer-1 antigen; iPSC, induced pluripotent stem cell; MINC, migration of neural crest cell; NCC, neural crest cell; NOAEL, no observed adverse effect level; PBS, phosphate buffered saline; PCB, polychlorinated biphenyl; PM, prediction model; p75, low affinity nerve growth factor receptor; ROI, region of interest

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of human cells has become possible due to stem cell technology that allows generation of specific cell types.

Testing of chemicals for developmental toxicity has a high priority, as an increase in the global incidence of developmental disorders was registered in the last two decades. In 2006-2010, the prevalence rate of congenital anomalies in Europe reached 2.4%, with one out of ten specifically related to defects of the nervous system (Dolk et al., 2010). The causes of developmental disorders are extremely heterogeneous. The anomalies can be directly caused by genetic alterations in the embryo, or they can be triggered by chemicals affecting embryonic development. Moreover, they may have an indirect origin associated with maternal exposure to external factors, such as infections, trauma or drugs. A better understanding of the developmental processes and how toxicants affect them is important to identify the causes and to develop strategies to avoid them (Barouki et al., 2012; Grandjean and Landrigan, 2014). This applies to a particularly high degree to the field of developmental neurotoxicity (DNT) (Bal-Price et al., 2012; Crofton et al., 2012).

Modern in vitro DNT assays are designed to model a specific biological key event (Leist et al., 2010; Crofton et al., 2011) at defined developmental stages (Stunnam et al., 2009; van Dartel et al., 2009; Zimmer et al., 2011; van Thriel et al., 2012). For instance, assays have been developed that assess changes in early neural differentiation (Balmer et al., 2012; Pennings et al., 2012; Rempel et al., 2015; Shinde et al., 2015), neurite outgrowth (Stiegler et al., 2011; Krug et al., 2013a), synaptogenesis (Harrill et al., 2011), gliogenesis (Fritsche et al., 2005) or myelination (Zurich et al., 2000, 2002).

A subset of biological key events relates to the specification, migration and differentiation of neural crest cells (NCCs). These specialized cells arise at the time of neurulation and migrate to their target sites in the body, where they give rise to very different cell types such as sensory neurons, glia, melanocytes, adrenal medulla and cranial cartilage/bones. When the finely regulated processes of NCC development and migration are altered, dramatic pathological consequences, called neurocristopathies, can be the result. For example, failure of the NCCs to colonize the gut leads to incomplete innervation of the gastrointestinal tract, as the enteric neurons and glia are derived from the neural crest. This results in Hirschsprung’s disease, which affects 1 in 5,000 newborns (Farlie et al., 2004). Another disorder related to the function of NCCs is Treacher-Collins syndrome with a frequency of 1 in 50,000 newborns (Trainor, 2010). Patients have malformed mandibles, ears and teeth as well as cleft palate and eye problems. The etiology of this disease is linked to a diminished number of NCCs migrating to the cranial structures (Trainor, 2010). Other pathologies involving the NC comprise spina bifida, cleft palate and CHARGE syndrome.

Besides genetic causes, neurocristopathies might be caused by chemicals, and several substances are known to interfere with NCC migration. They comprise the anti-epileptic drug valproic acid (found to interfere with NCC migration out of chick neural tube explants, Fuller et al., 2002) and the triazole fungicides (disturbing NC migration in rat embryos, Menegola et al., 2005). However, only few assays that test NCC function have been developed. Some of these tests use ex vivo neural tube explants (Fuller et al., 2002; Bergeron et al., 2013; Usami et al., 2014, 2015), but this only allows low throughput testing and the data-analysis is very time-consuming. Furthermore, the use of animal cells might not be relevant for human toxicity, as there are tremendous species differences in the timing of epithelial-to-mesenchymal transition, delamination and migration of NCCs as well as the closure of the neural tube (Theveneau and Mayor, 2012). Moreover, different species express different cell adhesion molecules during NCC development (McKeown et al., 2013). For example, chicken NCCs express cadherin 6B and later cadherin 7 before and after delamination, respectively, whereas in mouse cadherin 6 is expressed in both situations (Pla et al., 2001).

To overcome the above-mentioned limitations, we established an NCC migration assay (MINC assay) using human NCCs differentiated from embryonic stem cells (Zimmer et al., 2012). In this assay, NCC migration is measured as the number of cells repopulating a cell-free area produced by scratching with a pipette-tip. Several known developmental toxicants were positive in the original MINC assay. A screen of recently marketed drugs (Zimmer et al., 2014) and a further exploration of environmental toxicants (Dreser et al., 2015) identified additional NCC toxicants, such as polychlorinated biphenyls and interferon-beta.

Although a semi-automatic image quantification tool already has been established (Dreser et al., 2015), the throughput of the scratch assay is limited by manual image acquisition. Moreover, the assay is highly dependent on the experimenter’s skills to achieve reproducible scratch properties. Therefore, we have developed a new assay format to overcome these limitations. Instead of a linear scratch, a circular cell-free area is produced using silicon stoppers of defined size. Image acquisition and analysis are performed in an automated high-throughput manner. This new circular MINC (cMINC) assay was assessed for potential confounding factors that could affect its robustness. This required benchmarking against other readouts related to migration, as well as a careful evaluation of the role of cell proliferation and cell death during the assay time period. A prediction model for functional NCC toxicants was developed.

2 Results. Manuscript 1: Design of a high-throughput human neural crest cell migration assay to indicate potential developmental toxicants.
and FGF8 for another seven days, cells were live-stained for human natural killer-1 (HNK-1, monoclonal anti-human CD57/HNK-1 antibody, Sigma-Aldrich) and FACS-sorted for positive expression of HNK-1 and negative expression of Dll1 using a BD FACSAria IIIu (BD Biosciences). The sorted cells were expanded in N2-S medium, consisting of DMEM/F12 (Gibco) and supplemented with 100 μg/ml apotransferrin, 25 μg/ml insulin, 8.6 μM glucose, 100 μM putrescine, 30 nM selenite, 20 nM progesterone (all purchased from Sigma-Aldrich), 1x GlutaMax and 1% penicillin/streptomycin (both from Gibco), 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml FGF2 (both from R&D). Medium was changed every other day, and cells were passaged every 5-7 days by detachment with HyClone HyQtase (GE Healthcare Life Sciences). After 27-30 days, cells were cryopreserved at 4*10^6 cells/ml in 90% fetal bovine serum (FBS) (PAA Laboratories GmbH) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -80°C. Before use, 1x FBS was added and the cells were thawed and expanded in N2-S medium, consisting of DMEM/F12 (Gibco) and supplemented with 100 μg/ml apotransferrin, 25 μg/ml insulin, 8.6 μM glucose, 100 μM putrescine, 30 nM selenite, 20 nM progesterone (all purchased from Sigma-Aldrich), 1x GlutaMax and 1% penicillin/streptomycin (both from Gibco), 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml FGF2 (both from R&D). Medium was changed every other day, and cells were passaged every 5-7 days by detachment with HyClone HyQtase (GE Healthcare Life Sciences). After 27-30 days, cells were cryopreserved at 4*10^6 cells/ml in 90% fetal bovine serum (FBS) (PAA Laboratories GmbH) and 10% dimethyl sulfoxide (DMSO) (Millipore) until further use. For all migration experiments, freshly thawed NCCs were used and cultured in N2-S medium.

Tab. 1: List of chemicals tested in the cMINC assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tested concentrations</th>
<th>LOAEL migration 24 h</th>
<th>Stock concentration (solvent)</th>
<th>CAS #</th>
<th>Catalog #</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine</td>
<td>1.25 - 20 nM</td>
<td>10 nM</td>
<td>2mM (H2O)</td>
<td>64-86-8</td>
<td>C9754</td>
<td>Sigma</td>
</tr>
<tr>
<td>Taxol</td>
<td>0.3125 - 40 nM</td>
<td>10 nM</td>
<td>10 mM (DMSO)</td>
<td>33069-62-4</td>
<td>1097</td>
<td>Tocris</td>
</tr>
<tr>
<td>CytoD</td>
<td>12.5 - 400 nM</td>
<td>200 nM</td>
<td>5 mM (DMSO)</td>
<td>22144-77-0</td>
<td>C8273</td>
<td>Sigma</td>
</tr>
<tr>
<td>FBS</td>
<td>0.625 - 4%</td>
<td>2%</td>
<td>–</td>
<td>–</td>
<td>A15-101</td>
<td>PAA</td>
</tr>
<tr>
<td>As2O3</td>
<td>0.5 - 2 µM</td>
<td>1 µM</td>
<td>100mM (0.1% NaHCO3 in H2O)</td>
<td>1327-53-3</td>
<td>11099</td>
<td>Sigma</td>
</tr>
<tr>
<td>CdCl2</td>
<td>0.1 - 2 µM</td>
<td>0.5 µM</td>
<td>100mM (H2O)</td>
<td>10108-64-2</td>
<td>655198</td>
<td>Sigma</td>
</tr>
<tr>
<td>LCl</td>
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<td>10 mM</td>
<td>1 M (H2O)</td>
<td>7474-41-8</td>
<td>L9650</td>
<td>Sigma</td>
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<tr>
<td>Acrylamide</td>
<td>0.078 - 5 mM</td>
<td>2.5 mM</td>
<td>3.5 M (H2O)</td>
<td>79-06-1</td>
<td>A3553</td>
<td>Sigma</td>
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<tr>
<td>PCB180</td>
<td>1 - 20 µM</td>
<td>5 µM</td>
<td>20 mM (DMSO)</td>
<td>35065-29-3</td>
<td>35495</td>
<td>Sigma</td>
</tr>
<tr>
<td>Retinoic acid</td>
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<td>5 µM</td>
<td>100mM (DMSO)</td>
<td>302-79-4</td>
<td>R2625</td>
<td>Sigma</td>
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<tr>
<td>Aphidicolin</td>
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<td>1000 ng/ml</td>
<td>1 mg/ml (DMSO)</td>
<td>38966-21-1</td>
<td>A4487</td>
<td>Sigma</td>
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<tr>
<td>AraC</td>
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<td>100 mM (H2O)</td>
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<td>Sigma</td>
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<td>MG-132</td>
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<td>200 nM</td>
<td>10 mM (DMSO)</td>
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<td>S2619</td>
<td>Selleckchem</td>
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<td>Staurosporin</td>
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<td>40 nM</td>
<td>5mM (DMSO)</td>
<td>62996-74-1</td>
<td>19-123</td>
<td>Millipore</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>2.6 - 171 µM</td>
<td>43 µM</td>
<td>1.7 M (pure)</td>
<td>9002-93-1</td>
<td>93443</td>
<td>Sigma</td>
</tr>
<tr>
<td>AgNO3</td>
<td>3.125 - 100 µM</td>
<td>50 µM</td>
<td>1 M (H2O)</td>
<td>7761-88-8</td>
<td>7908.1</td>
<td>Roth</td>
</tr>
<tr>
<td>L-Homocysteine thiolactone</td>
<td>0.156 - 5 mM</td>
<td>2.5 mM</td>
<td>500 mM (H2O)</td>
<td>31828-68-9</td>
<td>H6503</td>
<td>Sigma</td>
</tr>
<tr>
<td>NaCl</td>
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<td>–</td>
<td>1 M (H2O)</td>
<td>7647-14-5</td>
<td>3957-1</td>
<td>Roth</td>
</tr>
<tr>
<td>DMSO</td>
<td>1%</td>
<td>–</td>
<td>–</td>
<td>67-68-5</td>
<td>1096780100</td>
<td>Millipore</td>
</tr>
<tr>
<td>EdU</td>
<td>10 µM</td>
<td>–</td>
<td>10 mM (H2O)</td>
<td>61135-33-9</td>
<td>part of kit</td>
<td>PanaTecs</td>
</tr>
</tbody>
</table>

Abbreviations: AraC, cytosine arabinoside hydrochloride; CytoD, cytochalasin D; EdU, 5-ethynyl-2'-deoxyuridine; LOAEL, lowest observed adverse effect level.
supplemented with 20 ng/ml EGF and 20 ng/ml FGF2. Exact procedures for the maintenance, differentiation and use of iPSCs are detailed in the supplementary file at https://doi.org/10.14573/altex.1605031s.

NCC migration setup

For all experiments, 96-well plates (Corning) were coated with 10 µg/ml poly-L-ornithine in 100 µl phosphate buffered saline (PBS) (PAA Laboratories GmbH) overnight in an incubator, washed three times with 120 µl PBS and further coated with 1 µg/ml fibronectin and 1 µg/ml laminin (both from Sigma-Aldrich) in 100 µl PBS. Plates were stored in the incubator until they were used, but at least overnight and not for more than three weeks.

Before seeding, the fibronectin-laminin solution was aspirated and the plates were dried for 15 min under sterile conditions. Silicon stops were inserted into the wells to create a circular cell-free area. Then, freshly thawed NCCs were seeded at a density of 30,000 cells per well (95,000 cells/cm²) in 100 µl medium. One day after seeding, migration into the cell-free area was initiated by manual removal of the stoppers and the medium was refreshed. For 48 h treatments, test chemicals were applied directly in fresh medium and then left for 48 h without further manipulation. For 24 h treatments, the compounds were added as 5x concentrate after additional 24 h of incubation, and overall migration was measured 24 h after addition of toxicants (i.e., 48 h after the medium change). Toxicants were diluted in medium and the final DMSO concentration did not exceed 0.1%. A list of the test chemicals and the concentrations used is given in Table 1.

Immunocytochemical characterization

For immunocytochemical staining, cells were seeded on plastic or glass, either evenly distributed at actual cell density (p75, GFAP, Pax6 staining) or as drops (20,000 cells per 10 µl drop) to observe migrating cells at the border (Nestin/F-actin co-staining). After one day in culture, cells were fixed with 4% formaldehyde, 2% sucrose in PBS or with ice-cold methanol. For intracellular epitope staining, cells were permeabilized for 15 min with 0.1% Triton and blocked with 10% FBS in PBS for 1 h before primary antibodies were applied in 4% FBS in PBS overnight. The next day, cells were washed with 0.05% Tween in PBS and secondary antibodies were applied (1:1000) for 1 h. Cell nuclei were counterstained with 1 µg/ml H-33342 for 10 min. Images were acquired using an Axio Observer.Z1 microscope (Zeiss, Oberkochen, Germany) equipped with an AxioCam MRm camera. The list of antibodies used is given at https://doi.org/10.14573/altex.1605031s.

For flow cytometric characterization, 120,000 cells were live stained for HNK-1 and p75. Cells were detached, counted and washed once in DMEM containing 10% FBS and subsequently washed in 2% of this mixture in PBS (washing buffer). Primary antibodies were applied for 30 min on ice, followed by two washing steps and incubation with appropriate secondary antibodies for 25 min on ice. After two more washes, cells were analyzed using a BD Accuri C6 flow cytometer (BD Biosciences).

In order to set the threshold for HNK-1 positivity, a set of cells was stained only with the secondary antibody. The threshold for positivity was based on the fluorescence intensity distribution of this population, so that 98.6% of this control population were defined as negative, and cells with a higher intensity than this threshold were defined as positive.

Image-based measurement of cell migration and viability

For flow cytometric characterization, cells were stained 47.5 h after initiation of migration with 1 µg/ml H-33342 and 533 nM calcein-AM (both from Sigma-Aldrich) and imaged 30 min later on a high content imaging microscope (Cellomics ArrayScanVTI, Thermo Fischer) equipped with a Hamamatsu ORCA-ER camera of 1024 x 1024 pixel resolution run at 2 x 2 binning mode. To obtain data on NCC viability, four fields outside the migration area were imaged with a 10x lens (approximately 500 cells/field). To obtain data on cell migration, the center of the well was imaged in four tiles with the 5x lens. The four images were jointed to obtain one micrograph covering an area of 2590 x 2590 µm.

Viability was defined as the number of H-33342 and calcein double-positive cells as determined by an automated algorithm described earlier (Stiegler et al., 2011; Krug et al., 2015). For quantification of migration, a software tool (freely available at http://invitotox.uni-koeln.de/) was developed to estimate the most likely position of the previously cell-free area (covered by the silicon stopper), to set thresholds for color intensity for both dyes, and to count the number of H-33342 and calcein double-positive cells in the region of interest (ROI). If treatment was for 48 h, the radius of the circular ROI was set at 1.0 mm. For 24 h treatments, the radius of the ROI was reduced to 0.7-0.9 mm to account for the fact that cells could migrate into the cell-free area for 24 h before the treatment was started. The radius of the reduced ROI was chosen in a way that at least 150 but not more than 300 cells were in the ROI in the untreated condition. A JPEG image of the final picture was generated to visually control the obtained result. The chosen settings were then transferred to all wells. Finally, the software counted the number of viable cells in the ROI for all wells of the plate and a data table was generated with the results. If not mentioned otherwise, viability and migration were normalized to the untreated control.

qPCR

Cells were seeded in 6-well plates at the same cell density as in the migration assay. Medium was exchanged after one day and cells were harvested after two further days and lysed in 1 ml PeqGOLD Trifast™ (PeqLab, Erlangen, Germany). Total RNA was isolated by phenol-chloroform extraction. Briefly, 200 µl chloroform was added, samples were vortexed and incubated for 3 min at room temperature. Then, samples were centrifuged at 4°C for 14 min at 20,000 g. The upper aqueous phase was transferred to a new vial and mixed with 500 µl isopropanol. After 10 min incubation at room temperature, samples were centrifuged at 4°C for 10 min at 20,000 g. The supernatant was removed and washed twice by carefully adding 1 ml of 75% ice-cold ethanol to the pellet followed by 5 min centrifugation at 10,000 g at 4°C. The pellet was dried for 30 min and 40 µl RNase-free water was added to the samples.
Reverse transcription was performed using 1 μg of RNA and the iScript™ Reverse Transcript Supermix (Bio-Rad, Muenchen, Germany) according to the manufacturer’s instructions. Reactions were performed in a volume of 20 μl, and 80 μl of RNase-free water was added afterwards.

Quantitative real-time PCR was carried out using 1 μl of the cDNA solution (with a concentration in the range of 0.1-1 μg/ml), 5 μl SsoFast™ EvaGreen® Supermix (Bio-Rad), 200 μM of each forward and reverse primer (sequences are given at https://doi.org/10.14573/altex.1605031s) in a total volume of 10 μl. Amplification was carried out in 96-well plates on a CFX96™ Real-Time PCR Detection System (Bio-Rad). Samples were heated for 2 min to 98°C, followed by 40 cycles of 2 s at 98°C and 5 s at 60°C. A melt curve from 75°C to 95°C in 0.5°C steps was obtained to measure the presence of primer dimers or other side products. The cycle threshold (Ct) values were determined using the Bio-Rad CFX Manager™ Software v2.0 (Bio-Rad). Results were analyzed using the 2ΔΔCt method (Livak and Schmittgen, 2001), i.e., Ct values of the genes of interest were compared to Ct values of the reference gene GAPDH.

Proliferation experiments
To measure proliferation, cells were treated as described in the section “NCC migration setup” with the exception that 10 μM 5-ethyl-2’-deoxyuridine (EdU), a thymidine analogue, was present during the treatment period. Proliferating cells incorporate EdU, which can later be detected using a click reaction. After imaging, cells were fixed with 4% formaldehyde and 2% sucrose (both from Sigma-Aldrich) in PBS for 20 min. The click reaction to detect EdU was performed according to the manufacturer’s protocol (EdU-Click 555, PanTecs, Heilbronn, Germany) without the permeabilization step and with washing performed with PBS only. Images were acquired using a high content imaging microscope (Cell Insight Personal Imager, Thermo Fischer) equipped with a QUANTIX camera of 1024 x 1024 resolution run at 2 x 2 binning. Proliferation was defined as the percentage of EdU positive nuclei among all H-33342 positive nuclei. As a positive control, cells were treated with 10 μM EdU and 1 μM cytosine arabinoside (AraC), a proliferation inhibitor. Migration was normalized to cells treated with EdU only for an overview of the correlation of EdU incorporation and migration inhibition.

To measure migration under proliferation inhibition, cells were treated as described in the section “NCC migration setup”, with the exception that 1 μM AraC was present during the treatment period. Migration was normalized to cells treated with AraC only.

Data handling and statistics
Values are expressed as means ± SD. If not indicated otherwise, experiments were performed at least three times (i.e., using three different cell preparations), with at least three technical replicates per condition. Statistical comparisons of selected experimental conditions vs controls (48 h cMINC assay) were done using an unpaired Student’s t-test. Values of p < 0.05 were considered statistically significant. Concentration-response curves from averaged data (n = 3 experiments) were fitted using R (R Core Team, 2015) and the package “drc” (Ritz and Streibig, 2005) to a log-logistic function with four parameters, where the upper asymptote was set to 100%, and appropriate constraints were used for the other parameters. The data points within a curve were weighted with 1/SD. EC90 and EC75 values of the fitted curves were estimated. If the estimated EC was above the highest tested concentration, this concentration was used for further calculations and the greater-than-sign was used. Additionally, “migration at the EC90 of viability” was estimated. If the EC90 was above the highest tested concentration, the extent of migration at the highest tested concentration was used and the smaller-than-sign was used.

3 Results and discussion
3.1 Characterization of the test system
Human NCCs were differentiated from pluripotent stem cells, based on an established protocol (Zimmer et al., 2012) (Fig. S1A, https://doi.org/10.14573/altex.1605031s). For most experiments, the human embryonic stem cell (hESC) line H9 was used. Alternatively, NCCs were generated from human iPSC experiments, the human embryonic stem cell (hESC) line H9 was used. For most experiments, the human embryonic stem cell (hESC) line H9 was used. Alternatively, NCCs were generated from human iPSC lines (Fig. S2, https://doi.org/10.14573/altex.1605031s). The protocol ended with an expansion phase of the NCC obtained by FACS sorting. During this time NCC characteristics were well retained as, e.g., measured by the expression of the NCC marker HNK-1 (Fig. S1B). This allowed the generation of large batches of cells that could be frozen and thawed as required for toxicity testing or further characterization of the cells under test conditions. Staining of the cryopreserved cells after thawing showed that ~95% of the cells were positive for at least one NCC marker (Fig. S1C). Immunostaining of cells plated for 24 h revealed that >99% were positive for the stem cell microfilament protein nestin (not shown), and that nearly all cells expressed the low affinity nerve growth factor receptor p75 (Fig. 1A). Further characterization showed that the NCC neither expressed the astrocyte marker GFAP nor the central nervous system precursor cell marker Pax6 (Fig. S1D). Co-staining for nestin and F-actin microfilaments revealed the mesenchymal-like cell morphology that is typical for NCCs and that clearly differentiates them from other related cell types (Fig. 1A).

Contact of cells to one another and to the extracellular matrix are important biological features in the context of NCC migration and its control. A major group of proteins responsible for matrix contacts are the integrins, while cadherins are key proteins determining cell-cell contact (Fig. 1B). The two groups of proteins are coded by a large family of genes, and the relative expression pattern of the respective family members is cell-type specific. Thus, the expression pattern of several integrins and cadherins was assessed by qPCR. A broad range of integrin subunits was expressed by the NCCs, and the expression pattern confirmed our earlier data obtained by microarray analysis (Zimmer et al., 2012). Of the β subunits, the highest mRNA-levels were of β1, but β3, β5 and β8 were also highly expressed. Of the α subunits, α1, α4, α5, α8, α11 and αV were highly expressed.
Of the tested integrins, only α6 and β2 were not detected. The subunit coded by β2 (CD18) is a typical leukocyte marker (e.g., also on microglia), and also the absence of α6 in NCCs is consistent with other findings, e.g., in the chicken α6 expression is downregulated at the onset of NCC migration and re-expressed upon neuronal differentiation (Bronner-Fraser et al., 1992; Testaz et al., 1999). The expression pattern quantified here indicates that the integrins could form heterodimers for several substrates, i.e., α1β1 and α11β1 for collagen substrates, α5β1, αβ3, αVβ5, αVβ8 for attachment to fibronectin and vitronectin, and also to some extent α3 and α7-containing dimers to bind laminin (Barczyk et al., 2010). In agreement with these results, the generated NCCs attached well to fibronectin, vitronectin and collagen and to some extent to laminin (data not shown). For the standard assay setup, a coating of poly-ornithine/fibronectin/laminin was used.

To the best of our knowledge, there is no information on integrin expression during human NCC development. However, integrin α4, which was highly expressed according to the qPCR data, has been reported to be expressed at high levels on NCCs of several animal species, and it has even been used as a specific NCC marker in mouse development (Kil et al., 1998; Testaz and Duband, 2001). Reports from different animal models also often described α5, αVβ1 and β3 on NCCs (Kil et al., 1998; Testaz et al., 1999; Haack and Hynes, 2001). Several studies in a variety of cell types have linked α4 and α5 to the regulation of cell migration (Mould et al., 1994; Beauvais et al., 1995; Wu et al., 1995); in particular, α4β1 is a typical integrin of highly migratory cell types (Sheppard et al., 1994; Haack and Hynes, 2001).

The more than 1000-fold expression difference between epithelial (E-cadherin, CDH1, low) and neuronal cadherin (N-cadherin, CDH2, high) indicated that the NCCs had undergone epithelial-to-mesenchymal transition (Nieto, 2011) and thus have a high migratory capacity. Other cadherins that showed high expression levels were CDH7, CDH10, CDH11 and CDH20 (Fig. 1B). The knowledge on cadherin expression by human NCC is extremely limited; moreover, pronounced species differences have been observed for this protein family (reviewed in Taneyhill, 2008; Strobl-Mazzulla and Bronner, 2012). Data from animal models suggest that emigrating NCCs upregulate...
Fig. 2: Assay principle and determination of cell viability

(A) For the standard assay setup, cells were seeded in wells containing silicone stoppers to create a cell-free circular area. One day after the seeding of the cells, the stoppers were removed and the cells were allowed to migrate into this circular area for 48 h. Then, the central circular area was imaged to assess cell migration, whereas images from the outer part were taken to measure cell viability. (B) To obtain data on the variability of plating and on the range of cell densities, the number of viable cells was counted (on day 2) in image fields of standardized size outside the migration area. Variations between plates, assays and cell batches are displayed. AU, arbitrary units (= cell count per unit area). (C) Schematic explanation of the algorithm used to evaluate cell viability: image information was used both from the H-33342 and the calcein staining pattern within the same image. In a first step, the H-33342 channel was analyzed, and the cell nuclei were segmented. Nuclei that either touched the border, were too small (nuclear debris of dead cells) or too bright (dying/apoptotic cells) were excluded (marked in orange). In a second step, information from calcein staining of the same field was used. The algorithm evaluates whether the calcein stain that co-localizes with the respective nuclear area is above a defined threshold, and thus the cell belonging to this nucleus is defined as viable (marked in blue). The pictures to the right are magnifications of the indicated cell area (in red) on the left. (D) Typical examples of image data related to viability measurements: untreated cells formed a dense cell layer, whereas cells treated with 100 or 200 µM chloroquine for 24 h (80% and 50% viable cells compared to untreated controls) were less dense and displayed more apoptotic nuclei.
CDH7 (in birds), CDH6 (in mouse), and CDH11 (in mouse and *Xenopus*) (Kimura et al., 1995; Nakagawa and Takeichi, 1995, 1998; Inoue et al., 1997; Vallin et al., 1998; Strobl-Mazzulla and Bronner, 2012; McKown et al., 2013). Moreover, CDH2, CDH6, CDH9 and CDH11 are expressed by mouse vagal enteric NCCs (Breau et al., 2006; Heanue and Pachnis, 2006; Vohra et al., 2006; McKown et al., 2013). This suggests that the human NCC generated here have a cadherin expression pattern consistent with their NCC identity, but it needs to be noted that different NCC populations in animals (along the rostral-caudal, and along the developmental timing axes) can have differing cadherin patterns.

In summary, this biological characterization of the test system, based on the set of cell adhesion molecules and the cytoskeletal phenotype, is well in agreement with the NCC being a migratory cell population suitable for test development.

### 3.2 Assay principle and determination of cell viability

The NCCs were used to establish a migration assay based on the principle that cells were plated around a silicone stopper in a culture dish and were allowed to move into the circular cell free zone (2 mm diameter) upon removal of the obstacle. After 48 h, the number of cells that had migrated into the circular target zone was quantified. Staining of cells with H-33342 and calcein-AM allowed counting of the total number of migrated cells. Moreover, recording of images in the same well but outside the migration zone allowed a robust and absolute (independent of control measures) quantification of the cell viability, e.g., after treatment of the cells with toxicants (Fig. 2A).

As the number of migrated cells depends on various assay conditions, such as the number of plated cells, it was interesting to obtain a measure of the variability of cell numbers in the assay. We observed up to twofold variations between cell batches (most likely due to the freezing conditions, and the differing proliferation rates of the lots). The variation of the number of plated cells between assays (using cells of the same frozen lot) was usually below 20% (due to, e.g., varying plating efficiency and small differences in proliferation during the assay time (72 h altogether)); the variation between plates within one assay was <5% (Fig. 2B), and the same was observed within one plate (not shown). This was deemed suitable for our approach of referring the number of cells in the migration zone to the number of cells in controls measured on the same plate, and in the same assay.

For the analysis of viability and migration, a previously published staining procedure as well as the cognate evaluation algorithm (Stiegler et al., 2011; Krug et al., 2013a; Hoehling et al., 2016) were adapted to NCC. Co-staining of nuclei with H-33342 and the overall cells with calcein-AM allowed segmentation and classification of the stained objects, as being: cell debris, dead cells (e.g., apoptotic cells) or viable cells (calcein-positive cell nuclei) (Fig. 2C). Untreated cells formed a dense cell lawn that was strongly calcein-positive. If cells were treated with a cytotoxicant, the number of stained cells decreased (Fig. 2D), and this loss could be quantified by using information from the H-33342 channel of the same field. The proposed setup thus allowed measurements of migration and viability in the same well.

This is important as small impairments of viability can seriously affect migration.

### 3.3 Generation of software for image processing related to the NCC migration assay

In our assay, migration was defined as the number of viable, i.e., H-33342 and calcein double-positive, cell nuclei in the migration zone. To obtain such data, a number of complex steps was required. To make this process at the same time traceable and less resource (operator time)-demanding, we developed a program with a graphical user interface (GUI) and automated standardized procedures that can be downloaded from our website (http://invitrotox.uni-konstanz.de/). The first problem solved by the software is the positioning of the “migration zone” in the image field. Alternative approaches would have been to assume the same central position (but in practice there is variation in the position of the silicone stoppers), or to image the cell layer before and after migration (but this would require fluorescent labelling of the cells before the assay and this manipulation might affect their function). After positioning of the region of interest (ROI, “migration zone”), the software counts the number of viable cells in this ROI. This process is supported by the GUI, which allows the user to set optimal parameters for image segmentation (Fig. 3).

This procedure allowed a half-automated evaluation of migration for a 96-well plate. Usually parameters were adapted for each plate, as the calcein signal sometimes varied between plates (e.g., due to incubation time). Importantly, the software allowed a quick visual control of all ROI on the plate. This was always used for a reality check and wells with badly positioned ROI were excluded. The latter happens, e.g., when cells are plated inhomogeneously or when the illumination of the imaging field is uneven. For practical purposes, it is also important to know that the segmentation fails if cells are plated too densely (overlapping nuclei of migrated cells) or if most of the cells in the migration zone are dead. Under such conditions, no reasonable migration data can be obtained from the assay, and the reality check by the operator, as offered by the software (required time = 1-2 min), can reduce the likelihood of artefactual data from technically poor assays. In a typical experiment, not more than five wells (<8%) were excluded per plate.

### 3.4 Preliminary evaluation of ring assay performance using exposure conditions (48 h exposure) similar to the scratch assay

In the previously published wound healing assay, cells were seeded and grown for two days before a scratch wound was introduced. Then toxicants were added and cells were allowed to migrate for 48 h (Zimmer et al., 2012; Dreser et al., 2015). We maintained the 48-h toxicant exposure period in the new assay format, but we introduced a small modification: to avoid well-to-well variations in the cell number at the start of the assay (due to 48 h of proliferation), we slightly modified the protocol by plating higher cell numbers (95,000 cells/cm² instead of 66,000 cells/cm²) and initiating toxicant exposure and migration one day after plating (Fig. S3A, https://doi.org/10.14573/altex.1605031s). The performance of the new migration assay, as to the detection of...
In a next step, two environmental toxicants known to inhibit NCC migration in the original MINC assay (Dreser et al., 2015) were tested. Both As$_2$O$_3$ and CdCl$_2$ inhibited migration in the high nM range, but it was not entirely clear whether this effect could be distinguished from general cytotoxicity (Fig. S3B). In this field, the most likely original stopper position (= “migration zone”) was determined automatically by an algorithm that searched for the minimum of signal intensity in the green channel. In a third step, image features were optimized for this migration zone in a user-guided semi-automated way. For instance, threshold and brightness were adjusted manually for both fluorescence channels in a control image. The parameters were considered optimal if each viable cell (green) contained a recognized nucleus (red). The same parameters were then applied by the software to the images from all wells, and the individual cells were identified and quantified automatically (using standard segmentation procedures). Cell debris and nuclear fragments of dying cells were excluded automatically from analysis by setting a minimal threshold for nuclear size.

In a next step, two environmental toxicants known to inhibit NCC migration in the original MINC assay (Dreser et al., 2015) were tested. Both As$_2$O$_3$ and CdCl$_2$ inhibited migration in the high nM range, but it was not entirely clear whether this effect could be distinguished from general cytotoxicity (Fig. S3B). For all data points at which migration was reduced by more than 25%, viability was reduced by more than 10%. Possibly, different choices of test concentrations may have identified a narrow concentration range in which specific effects on migration occur (> 25% reduction at > 90% viability). For the purpose of the initial evaluation it was sufficient to see that the toxicants showed some tendency to be detected, but were not easily (broad effective concentration range) identified. Note that data stems from three independent experiments.

Fetal bovine serum was found to increase migration in a concentration-dependent manner (Fig. S3C), indicating that the...
assay is capable of identifying not only migration-inhibiting but also migration-promoting agents. As a further mechanistic test compound, we used the cytostatic drug AraC to investigate effects of toxicants that affect cell proliferation. AraC affected the migration endpoint (apparently reduced migration), but it also reduced the overall number of viable cells. This indicated that compounds that strongly affect cell proliferation may appear as migration inhibitors and require special attention (Fig. S3C).

Finally, two typical toxicants were tested that have not been used in the MINC before, but where literature data indicate that they could affect NCC migration: acrylamide affected the function of immature neurons derived from NCC (Hoetling et al., 2016) and may therefore also affect NCC function; LiCl affects the migration of other cell types (Wang et al., 2013; de Araujo et al., 2016) and it was therefore a candidate for effects in the MINC assay. In fact, the environmental toxicant acrylamide was specifically migration-inhibiting at concentrations around 1 mM (similar to its effects on neurons), and lithium chloride, a drug used for mood disorders, inhibited migration at a concentration of about 10 mM, similar to its inhibitory effect on glycogen synthase kinase-3 (Bain et al., 2003; Selenica et al., 2007) (Fig. S3D).

The initial evaluation showed that the new test system is able to detect migration-modulating compounds, but for some compounds there may be only a narrow concentration window to identify specific effects. Furthermore, the data obtained with the proliferation inhibitor AraC suggested that the issue of cell proliferation deserved further scrutiny.

### 3.5 The role of cell proliferation in the 48-h exposure protocol

To investigate proliferation in the 48-h setup in more detail, NCCs were exposed to EdU during the migration phase. This thymidine analog is incorporated into the DNA of proliferating cells, and it can be easily visualized by coupling to a fluorophore. This EdU stain, when combined with H-33342 (staining all nuclei) allowed the discrimination of cells that had undergone replication (S-phase) during the period of EdU exposure (double-positive) and cells that had not proliferated. We found that approximately half of the cells were EdU-positive after 48 h incubation (Fig. S4A, https://doi.org/10.14573/altex.1605031.s). An exact quantification (eight separate experiments, based on four different cell lots) showed that 61.6 ± 10% of the cells (means ± SD) were EdU-positive (Fig. S4B). This means that up to 45% of the NCCs present at the beginning of the assay proliferated during the 48-h assay period (the mathematical calculation of this number is based on the assumption that each cell division yields two EdU-positive cells from one original cell).

This data, based on EdU incorporation is consistent with our data on cell doubling time (about 70 h) obtained by cell counting. The contribution of cell proliferation to the number of cells in the migration area provides a strong biological rationale for potential misclassification of compounds that affect cell proliferation.

To test directly whether toxicants affected proliferation at concentrations that apparently reduced migration, we measured their effect on EdU-incorporation. For instance, LiCl did not affect EdU incorporation, whereas As2O3 and CdCl2 both reduced EdU incorporation by about 40% (Fig. S4C). We also tested whether the apparent enhancing effect of FBS on migration may be due to the stimulation of proliferation. However, this was not the case (Fig. S4C).

Having established that some compounds do inhibit proliferation, and may therefore be falsely classified as migration inhibitors, we used a different technical approach to directly assess mitosis events in the migration zone: cells were observed by video time-lapse microscopy in the migration zone. The two migration-inhibitors used as positive controls, cytochalasin D and taxol, did not significantly affect proliferation, and neither did LiCl. In contrast, CdCl2 treatment significantly reduced replication by about 40%. This corroborated the data obtained with population-wide measurement of EdU incorporation. Treatment with the anti-mitotic agent AraC blocked proliferation nearly completely. Only 3.1% of cells arose from mitosis under this condition, and all the observed cell divisions happened in the first 18 h after AraC addition (data not shown), indicating that these cells probably had started proliferation before AraC addition. Addition of FBS led to a slight, non-significant increase in the proliferation rate. This observation also corroborated the data obtained from EdU incorporation experiments (Fig. S4D).

We conclude from these observations that the cMINC can give alerts on compounds that impair NCC mobility; but our experiments (measurement of EdU incorporation; addition of AraC) also showed that cell proliferation contributes up to 25% of the cells in the migration zone. Therefore, toxicants that affect proliferation, but not migration, may be falsely classified as migration inhibitors. In the following, the protocol was modified to render the assay more specific.

### 3.6 Optimization of the cMINC assay protocol

To reduce the effect of toxicants on viability and cell proliferation, a shorter toxicant exposure would be favorable. Therefore, we explored a new exposure scheme that exposed the cells to toxicant only during the last 24 h of migration (Fig. 4A). Under these conditions, as before, cytochalasin D and taxol reduced the number of cells in the migration zone and FBS increased it (Fig. 4B). Alternatively, cells were exposed during the first 24 h of migration, and the assay was ended by measurement of viability/migration. This modification did not improve the assay results and was not pursued further (data not shown).

During the optimization experiments, it became evident that there was always a particularly high cell number at the outer rim of the migration zone. We hypothesize that these cells entered the ROI either (i) by “free” migration, (ii) by being “pushed” by the expanding cell layer outside the zone, or (iii) by proliferation of cells bordering the migration zone. Only the first driving force was relevant to our test endpoint, while the other two increased the level of the background signal. Therefore, it was desirable to “cut” the outermost rim from the migration zone. This was achieved by introducing a feature to our quantification software that allows choosing the radius of the migration zone. As the radius of the original silicone stopper was 1 mm, choice of a migration zone radius of 0.9 mm cut out the outermost rim (Fig. 4C). In a control experiment (untreated cells), we tested the influence of radius settings on the number of cells detected in the ROI after 24 h and 48 h. After 24 h of migration, approxi-
2.3 Results and discussion

approximately 400 cells were in the 1 mm radius ROI. When the radius of the ROI was reduced from 1 mm to 0.7 mm, only few cells were present in the ROI. This number then increased strongly during the following 24 h (Fig. 4C, red line). Also, when the radius was only reduced by 100 µm (Fig. 4C, blue line), less than 200 cells were present in the ROI after 24 h, and this number increased steeply (3-fold) within the next 24 h. Thus, reduction of the radius of the ROI allowed elimination of cells entering the migration zone unspecifically, and it allowed a focus of the assay on cells that entered especially during the last 24 h (i.e., during the treatment period). For all following experiments, the size of the ROI was chosen so that it contained at least 150 but not more than 300 cells for control conditions. This radius setting (mostly using values of 0.7-0.85 mm) was done in the same way for all wells of a plate after initial settings by the operator on the GUI.

Using this new setup, concentration-response curves were obtained for several groups of compounds (Fig. 4D-E and Fig 5). In agreement with the results of 48 h exposure, cytochalasin D...
and taxol inhibited migration and FBS increased migration (Fig. 4D), although FBS was less effective than in the 48-h setup. Colchicine, a microtubule polymerization inhibitor also inhibited migration in the nM range. (Fig. 4D). The cytostatic drug AraC had less pronounced effects than in the 48-h setup, and it would now definitely not be scored as a migration inhibitor.

The obtained results were verified by time-lapse video microscopy and tracking of individual cells (Fig. S5, https://doi.org/10.14573/altex.1605031s). Cytochalasin D and taxol both decreased the distance covered by cells within 24 h (i.e., the speed of individual cells), whereas FBS had the opposite effect. The proliferation inhibitor AraC did not affect cell speed. Thus, the different analytical approach of direct tracking of migrating cells over the full assay time fully corroborated the results obtained with the “fixed time point” cMINC assay.

The negative controls NaCl, DMSO as well as EdU did not affect viability and migration (Fig. 4E).

Testing of a variety of developmental toxicants under the new cMINC conditions suggested that As$_2$O$_3$ and CdCl$_2$ inhibit migration; this effect was more distinct than in the 48-h setup (Fig. 5A). Acrylamide and LiCl had a wider concentration window, but the potentially specific concentration range of acrylamide was still narrow compared to that of CdCl$_2$ or LiCl. Polychlorinated biphenyl (PCB)180, a known NCC migration inhibitor (Dreser et al., 2015), strongly inhibited migration in this setup (even more effectively than cytochalasin D and taxol). Further-
more, another migration-inhibiting compound was identified: the teratogen retinoic acid inhibited NCC migration at µM concentrations (Fig. 5A). Similar data were obtained with cells of different origin, such as human iPSC lines (Fig. S2).

Finally, we explored the effect of unspecific toxicants on the test system endpoints. The cytotoxicants Triton X-100 (detergent), AgNO₃ (toxic metal), and L-homocysteine thiolactone (toxic metabolite found in human neurological disorders) reduced the migration endpoint, but they also reduced cell viability at similar concentrations (Fig. 5B). From the comparison of the endpoint curves, we would conclude that such compounds are not specific inhibitors of NCC migration. We also tested another proliferation inhibitor, the DNA synthetase inhibitor aphidicolin (Fig. 5B). Like AraC, it only produced a minor effect. Other cytotoxicants tested were the proteasome inhibitor MG-132 (Grimm et al., 1996; Qiu et al., 2000) and the universal apoptosis inducer staurosporine (Weil et al., 1996; Nicotera and Leist, 1997). Here, the concentration-response curves of viability and migration also separated only to a small extent (Fig. 5B).

In conclusion, the new 24 h cMINC assay protocol allowed a better separation of migration inhibitors from compounds that indirectly affected migration through effects on viability and/or proliferation.

### 3.7 The role of proliferation for assay outcome in the optimized cMINC

The fact that AraC affected migration also in the 24-h setup suggested that proliferation still played a role. To quantify this, EdU incorporation was measured, and about half of the cells (exactly 52%) in the migration zone were found to be EdU positive (Fig. 6A). This means that one third of the cells that entered the migration zone during the assay divided during the 24 h of the assay period. Moreover, this means that only 75% of the cells present at the end of the assay were present before toxicant addition (Fig. 6B). If a compound inhibited all proliferation (like AraC) it would thus reduce the final cell number by 25%. This would be measured as a viability of 75% (number of cells compared to untreated controls), and as an inhibition of migration by 25% (i.e., a cell number in the migration zone of 75%, compared to controls), in parallel with the overall 25% reduced cell number.

This consideration is corroborated by experimental data obtained from cells treated with AraC. Exposure of NCCs for 24 h with 1 µM AraC resulted in 80% viability and approximately 75% migration (Fig. 4D). Thus, a reduction of migration by > 25% is unlikely to be explained by effects on proliferation.

We used a large variety of treatment conditions to explore whether there was any correlation of reduced proliferation with reduced migration in the cMINC assay. EdU was added during the treatment period. At the end of the test, viability and migration were measured following the standard protocol, and afterwards cells were fixed, stained and scored for EdU (in the cell layer outside the migration zone). Proliferation and migration did not correlate (Fig. 6C). Three different scenarios were observed: (I) conditions that inhibited proliferation but not migration. Compounds in this group comprised the proliferation inhibitors AraC and aphidicolin as well as MG-132 and staurosporin (Fig. 6C, group I in black); (II) Conditions that inhibited migration but not proliferation. This group comprised cytochalasin D, taxol as well as PCB180 (Fig. 6C, group II in blue); (III) conditions that affected both proliferation and migration. High concentrations of LiCl, CdCl₂, and acrylamide and low concentrations of colchicine fell into this third group (Fig. 6C, group III in red).

For the last group of conditions (group III), it cannot be ruled out that the observed inhibition of migration is caused partially by inhibition of proliferation. To get more clarity on the effects of such compounds, the cMINC was performed in the presence of AraC in controls and in all treated samples to eliminate proliferation. Aphidicolin was measured under the same conditions as an example for group I. There was no effect on viability or migration (Fig. 6D), in contrast to data obtained in the absence of AraC (Fig. 5B). This showed that the apparent cytotoxicity of aphidicolin under control conditions was in fact due to a diminished cell number as a result of reduced proliferation. Cytochalasin D, a compound of group II, also inhibited migration when co-treated with AraC (Fig. 6D). Therefore, cytochalasin D can be considered a “true migration inhibitor”, i.e., acting also under conditions when proliferation does not play a role. Such data might suggest that the cMINC should always be performed in the presence of AraC. This altered protocol may result in the reduction of noise, an improved baseline, and the avoidance of artifacts caused by compounds that attenuate cell proliferation. However, it may also be argued that such a test setup also has disadvantages, as cells would be exposed to a known toxicant (AraC), and there may be interactions between this compound and the test compounds that cannot be anticipated. Our suggestion for a testing strategy is to perform the cMINC without addition of AraC for the initial screening of compounds. Problematic compounds (unclear effects; only partial separation of viability and migration curves; evidence or suspicion of inhibited proliferation) would then be re-tested, e.g., in the modified cMINC in the presence of AraC. Such a sequential approach is in agreement with the concept that the results from a screening assay (as the cMINC) can never be taken as definitive toxicological proof without further confirmation.

We tested such an approach, using some of the compounds in group III (ambiguous conditions). Acrylamide, CdCl₂, LiCl and colchicine all inhibited migration in the cMINC with AraC in at least one concentration that did not reduce viability (below 90%). For instance, 20 mM LiCl, a condition that gave ambiguous results in the absence of AraC (Fig. 5A), clearly inhibited migration in the AraC-modified cMINC without causing cytotoxicity (Fig. 6D). Potency and efficacy of some compounds were altered by testing in the presence of AraC. As these shifts were relatively moderate, we consider this an additional reason to use AraC-free conditions for standard testing, and follow up with an AraC-modified cMINC when inhibition of proliferation is suspected (e.g., reduction of cell number at the end of the assay without evidence for cell death).

### 3.8 Construction of preliminary prediction models for the optimized cMINC test

During test development, we used semi-quantitative estimates of test outcome, such as visual comparisons of curve shapes or positioning of point estimates of data relative to benchmark
Fig. 6: The role of cell proliferation in the new cMINC assay format
Cells were plated (30,000/well) and migration was initiated by removal of the stoppers one day later (as in Fig. 4A). After 24 h, test compounds were added and assay endpoints were assessed 24 h thereafter. (A) Cells were incubated with EdU (10 µM) during the last 24 h of the assay, and the number of EdU and H-33342 double-positive cells was quantified (means ± SD; n = 3). (B) A schematic diagram illustrates the relation of dividing cells and cells incorporating EdU: 50% of EdU positive cells will arise if one third of the cells divides over a 24 h period. (C) Relationship between cell proliferation (assessed by the incorporation of EdU (10 µM)) and apparent cell migration (assessed in the cMINC assay): some exposure conditions inhibited proliferation but not migration (upper bar graph, black group in scatter plot). Under some conditions, migration was inhibited, but not proliferation (lower bar graph, blue group in scatter plot), and some affected both endpoints (red group). (STS, staurosporine; MG, MG-132; Aph, aphidicolin; AraC, cytosine arabinoside) (D) Concentration-response curves for viability and migration in the presence of the proliferation inhibitor AraC (1 µM) for selected compounds of the three different groups in (C). All values are normalized to cells treated with 1 µM AraC only. Data are means ± SD from three independent experiments.
values (90% viability; 75% migration). Finalization of test development and objective classification of compounds requires a prediction model. A suitable prediction model has to take into account that the cMINC assay measures two endpoints simultaneously: migration and viability. One solution for other assays with two endpoints (e.g., neurite outgrowth) has been to calculate the ratio between effective concentrations (EC) of both endpoints (Stiegl et al., 2011; Krug et al., 2013a; Hoelting et al., 2016; Ryan et al., 2016). For example, Stiegl et al. used the ratio of EC50 of viability to EC50 of neurite outgrowth; Ryan et al. used the ratio of benchmark concentrations (BMC) for viability to neurite outgrowth. Although this approach has proven beneficial in these cases, it is less suitable for the cMINC assay for several reasons: (i) some compounds do not affect viability at all in the testable concentration range; (ii) some compounds substantially affect migration, but the determination of a meaningful EC50 value is problematic for mathematical reasons because of the curve shape; (iii) migration requires the contribution of multiple signaling pathways, and inhibition of any one of them only partially impairs cell migration (Zimmer et al., 2012). This results in curves that have asymptotes at, e.g., 70, 50 or 30% migration capacity, or that do not have an asymptote at all, as increasing toxicant concentrations activate different mechanisms of toxicity. This uncertainty in curve fitting may create uncertainties for benchmark values also in the upper part of the curve (e.g., for determination of BMC10 or BMC15 values). We therefore decided to establish various candidate prediction models (PM), to compare their performance, and to pick the best-suited PM for more extensive evaluation in follow-up studies.

We used sixteen compounds for the initial evaluation. These comprised 9 positive controls and 7 compounds assumed to have no specific effect on migration (unspecific controls). A good PM would classify the 9 positive controls as “specific migration inhibitors” and the other seven compounds as “non-specific toxicants”. We used fixed benchmark values of 90% and 75%. The 90% cutoff is a commonly used value, based on biological plausibility that changes in a viability parameter below this value may not be meaningful. The 75% cutoff for migration is based on our findings that artefacts due to inhibition of proliferation may cause up to a 25% change of migration, but not more (Fig. 6). An alternative approach to the choice of fixed values would have been a determination based on the noise of the background signal (e.g., 3 x SD of solvent controls). In practical terms, such an approach (Ryan et al., 2016) would have yielded relatively similar cutoffs, not far from the 90% and 75% values. We preferred the fixed value approach here for its anchoring to biological plausibility, instead of test statistics. For future determinations of a definite PM model, both approaches will be reconsidered.

First, we fitted curves through the viability and migration data and determined an EC90 value, i.e., an effective concentration bringing viability down to 90% (EC90V) and an effective concentration bringing migration down to 90% (EC90M). Ranking the compounds according to the ratio EC90V/EC90M did not allow separation of specific and unspecific compounds. Also, other EC-based ratios (EC75V/EC75M; EC50V/EC50M) were not useful (Fig. 7A).

Second, we proceeded as in the first approach, but used different benchmark values for migration and viability (e.g., 75%/90%). On this basis, various ratios were calculated. For instance, the EC90V/EC75M was used. In this model, a ratio of 1 corresponds to 25% migration inhibition at 90% viability. Compared to the first approach, this PM takes into account that BMCs for different endpoints may be different due to the difference of the underlying biological process or due to differences in baseline noise. Using the EC90V/EC75M ratio allowed separation of specific and unspecific compounds (Fig. 7B). All unspecific compounds had a ratio ≤ 1.11, whereas calibration compounds and developmental toxicants reached a ratio of > 1.3. Thus, the EC90V/EC75M ratio allowed a separation of the two compound groups, and a threshold value of 1.3 may be used for classification. However, the obtained parameter is unit-less and does not contain information about the efficacy of migration inhibition.

As a third approach, we examined the level of migration inhibition at the no observed adverse effect level (NOAEL) of viability (NOAELV). This method was used in the previously published wound healing assay (Zimmer et al., 2012). However, the NOAEL depends on the spacing of the data points and hence is not always accurate. To circumvent this problem, we slightly modified the approach and used the level of migration inhibition at the EC90M. This PM also allowed separation of the two compound groups (Fig. 7C). All calibration compounds reached at least 40% of migration inhibition whereas the unspecific compounds only inhibited migration up to 30%. Thus, a threshold value for classification may be set at 30%, but better definitions are possible after testing of a larger group of compounds. Alternatively, a threshold may also be set at 25% migration inhibition. This would correspond to the rough threshold setting in the initial part of our study. Such a decision would decrease the specificity of the assay, but increase its sensitivity. If the assay is performed for screening or to obtain alerts on potential developmental toxicants, we suggest to use the 25% threshold and to follow up with a secondary test.

Complementary to the PM, a ranking of test compounds according to potency is useful for some applications. An overview of the EC90V values (Fig. 7D) shows that our study was based on a wide dynamic range (six orders of magnitude) of toxicities and still distinguished specific and unspecific migration inhibitors over the full range of general cytotoxicity. As a measure for potency with respect to migration inhibition, we propose the use of the EC75M (Fig. 7E). Two considerations are important in this context: first, the measure of “migration potency” is only meaningful for compounds that have been defined as migration-inhibitors in a prediction model (e.g., > 25% migration inhibition at the EC90M), and it should not be used for other compounds; second, we decided here to use EC75M instead of, e.g., EC70M. This follows the precautionary principle of assuming that biologically meaningful effects may already occur when migration is inhibited by 25%.

For further use, we suggest to use both the “EC90V/EC75M” PM and the “migration at EC90V” PM, and to combine them by defining a hit as when a compound is classified as positive in either of the models. A hit would be followed up by secondary testing (concentration response curve in cMINC with more data...
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Fig. 7: Preliminary prediction model to classify tested compounds in the cMINC assay
Assay setup and data acquisition were as in Fig. 4; migration (M) and viability (V) were measured for 16 compounds. Endpoint-specific controls are depicted in turquoise, positive control test compounds in blue and negative/unspecific compounds in red. (A, B, C) Schematic representation (on the left) of the parameter used to rank the compounds and the corresponding ranking (on the right). (A) Ratio of EC90 of viability and EC90 of migration (EC90_V = concentration with 90% of normal cell viability; EC90_M = concentration allowing 90% of the full migration). (B) Ratio of EC90 of viability and EC75 of migration (EC75_M = concentration allowing 75% of the full migration). (C) Migration inhibition (= % reduction of migration) at the EC90 of viability or at the no observed adverse effect level (NOAEL) of viability. Note that “adverse” is defined here as reduction of viability by > 10%. (D) Graphical representation of the potency of compounds with respect to effects on cell viability (as EC90 of viability). The asterisk (*) indicates that the real EC90 is higher than the value shown; in these cases, the highest concentration tested is displayed. (E) Graphical representation of the potency of compounds with respect to effects on cell migration (as EC75 of migration).

points; AraC-modified cMINC), a definition of potency, and an estimate of efficacy. To further reduce uncertainty, additional types of migration assays may be used in the future.

4 Conclusions

We successfully established an assay to test for interference with human NCC migration. Proliferation was identified as a potential confounding factor, which can be overcome by repetition of the assay under conditions suppressing proliferation (presence of mitotic inhibitors). Furthermore, we compared the standard migration endpoint with information from video microscopy time-lapse experiments. We established a preliminary prediction model on the basis of these data. The assay has a medium to high throughput (The automated image acquisition and the analysis with a tailored software allow a skilled operator to measure 500-1000 wells/week. This would yield 100-200 data points/week assuming the use of five technical replicates). However, the production of sufficient cells for high throughput testing would be limited by the laborious standard differentiation protocol. An alternative procedure to differentiate NCCs (Mica et al., 2013), as used here for one of the iPSC cell lines, allows generation of NCCs in significantly shorter time.
Concerning the biological relevance of the test system, it needs to be noted that migration occurs in vivo in a 3D cellular environment: NCCs perceive chemoattractive and chemorepulsive signals from the surrounding cells and permeate other tissues/cell populations during their migration on defined routes (Theveneau et al., 2013; Green et al., 2015; Shellard and Mayor, 2016). In contrast to this, our test uses a 2D cell free migration zone. It is mechanistically not fully understood why the cells migrate into the cell-free space. One hypothesis is that the underlying mechanism is a non-directed cellular drive for movement. Apparently-directed movement into the migration zone may occur because there are fewer cells in this circular area and thus fewer contact-inhibition signals are perceived. Another hypothesis is that the cells migrate via a “chemotactic mechanism”, i.e., that they perceive signal differences between the cell-free space and the populated area. So far, information from the time-lapse experiments agrees best with the first hypothesis, as the cells do not seem to migrate in a directed manner into the cell-free circle. In the future, it would be interesting to model the chemotactic migration of NCCs more closely, and to investigate whether toxicants are able to specifically alter chemotaxis of NCCs.

The classical animal-based approach utilized for developmental toxicity testing comprises the measurement of apical endpoints such as tissue malformations, mortality and growth retardation. This approach has a low sensitivity and specificity with respect to human hazard prediction (Hartung and Leist, 2008; Leist et al., 2008; van Thriel et al., 2012; Smirnova et al., 2014). The evaluation of in vivo developmental toxicity testing data indicates a high percentage of false positive (~40%) (Hartung, 2009) and of false negative (~55%) classifications (Bremer and Hartung, 2004). Furthermore, the concordance among different laboratory mammalian species is lower than 60% (Hartung, 2009; Sipes et al., 2011; Leist et al., 2014).

Some of these limitations may be overcome by human-cell based assays, as presented here (cMINC assay) or suggested earlier (Hansson et al., 2000; Scholz et al., 2011; Balmer et al., 2012). At present, the assay shares an important shortcoming with classical animal-based tests: it does not give information on the mechanism of migration inhibition. Toxicants could alter migration via different mechanisms, for example by interfering with cytoskeletal remodeling (i.e., cytochalasin D or taxol), by energy depletion (Völbracht et al., 1999; Latta et al., 2000) or by induction of cell differentiation. Moreover, it remains open whether the identified toxicants inhibit migration via a NCC-specific or a cell-type unspecific mechanism. Addressing such questions will require the combination of mechanism-oriented endpoints (e.g., omics technologies) with our phenotypic test, and extensive comparison to other models.

**Conflict of interest**

The authors declare no conflicts of interest.

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Combination of multiple neural crest migration assays to identify environmental toxicants from a proof‑of‑concept chemical library

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Abstract Many in vitro tests have been developed to screen for potential neurotoxicity. However, only few cell function-based tests have been used for comparative screening, and thus experience is scarce on how to confirm and evaluate screening hits. We addressed these questions for the neural crest cell migration test (cMINC). After an initial screen, a hit follow-up strategy was devised. A library of 75 compounds plus internal controls (NTP80-list), assembled by the National Toxicology Program of the USA (NTP) was used. It contained some known classes of (developmental) neurotoxic compounds. The primary screen yielded 23 confirmed hits, which comprised ten flame retardants, seven pesticides and six drug-like compounds. Comparison of concentration–response curves for migration and viability showed that all hits were specific. The extent to which migration was inhibited was 25–90%, and two organochlorine pesticides (DDT, heptachlor) were most efficient. In the second part of this study, (1) the cMINC assay was repeated under conditions that prevent proliferation; (2) a transwell migration assay was used as a different type of migration assay; (3) cells were traced to assess cell speed. Some toxicants had largely varying effects between assays, but each hit was confirmed in at least one additional test. This comparative study allows an estimate on how confidently the primary hits from a cell function-based screen can be considered as toxicants disturbing a key neurodevelopmental process. Testing of the NTP80-list in more assays will be highly interesting to assemble a test battery and to build prediction models for developmental toxicity.

Keywords Cell migration · Cell tracking · Cytotoxicity · High content imaging · Developmental toxicity · Human stem cells

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| hESC | Human embryonic stem cell |
| logP | Octanol–water distribution coefficient |
| MeHgCl | Methylmercury(II) chloride |
| MINC | Migration of neural crest cell |
| MW | Molecular weight |
| NCC | Neural crest cell |
| NHBA | Number of hydrogen bond acceptors |
| NOAELf | No observed adverse effect level for viability |
| NRB | Number of rotatable bonds |
| NTP | National Toxicology Program |
| PAH | Polycyclic aromatic hydrocarbon |
| PBDE | Polybrominated diphenyl ether |
| PBS | Phosphate buffered saline |
| PCA | Principal component analysis |
| QSAR | Quantitative structure-activity relationship |
| ROI | Region of interest |
| TB-BPA | Tetra(1,1,1-trifluoro-2,2-difluoroethyl)phenol A |
| TPSA | Polar surface |

Introduction

During the past 10 years, many new assays have been developed to identify developmental toxicants and developmental neurotoxicants (Bal-Price et al. 2012; Robinson and Piersma 2013; Schmidt et al. 2016; Smirnova et al. 2014; van der Laan et al. 2012). For such non-animal test methods, there are two fundamentally different applications: the first is for hazard identification of defined compounds, and potential further use of the data in risk assessment; the second is for screening of larger sets of compounds to identify those that require further testing. The latter approach requires a relatively high throughput, but has little stringent requirements for specificity and formal test validation. Examples for large screening programmes are ToxCast and Tox21 (Judson et al. 2013; Tice et al. 2013).

There has always been a strong focus of safety sciences on in depth characterization of individual compounds, either to elucidate mechanisms of toxicity or to provide sufficient data for regulatory purposes. In contrast to this, the idea of screening large numbers of compounds is a relatively new development (Collins et al. 2008; Leist et al. 2008; Shukla et al. 2010). It has become possible through adoption of high-throughput technology originally developed for drug discovery in pharmaceutical industry. Such screen data are frequently linked to large uncertainties. Moreover, the assays are generally established in a way that allows for many false-positives. Therefore, different strategies have been developed in the pharmaceutical industry to further filter, confirm and characterise the hits. A present challenge in toxicology is to establish corresponding quality evaluation and follow-up strategies for toxicity screens (Judson et al. 2013; Leist et al. 2014; van der Burg et al. 2015).

For drug discovery screens, the first step is invariably a confirmation testing based on the same assay as used for initial screening. In general, this is followed by secondary, tertiary and higher level tests to reduce the level of technical and biological uncertainty. Such assays use different technologies, different analytical readouts or other biological test systems. Moreover, the follow-up tests often increase in complexity to get closer to providing human relevant information. In parallel, investigations are initiated to provide answers for the question on how broadly applicable a screen finding is, with respect to cell types, tissues, developmental stages and other important biological determinants. Although a lot can be learned from drug discovery, the development of own strategies for toxidology is important, because the objectives of safety sciences and drug discovery are different.

One approach of in vitro toxicology is to use several parallel tests (test batteries) instead of a single test. This provides information on broader sets of biological functions or pathways that may be disturbed. This principle has been used successfully for the definition of non-genotoxicants. If a compound is found negative in three non-animal tests that cover different types of biological processes relevant to DNA damage, then the chemical is judged to be non-mutagenic (Adler et al. 2011; Combes et al. 2008). Recently, also a test battery for dermal sensitization has been approved. In this case, the three tests currently forming the battery cover key events of an adverse outcome pathway (Baskettet et al. 2015; Ezendam et al. 2016; Reisinger et al. 2015). A third successful example of in vitro test battery use, in another domain of toxicology, is the potential substitution of the in vivo uterotrophic assay of the endocrine disruptor screening program by a set of ToxCast assays directed to various steps relevant for oestrogen signalling (Browne et al. 2015).

In the fields of neurotoxicity and developmental neurotoxicity (DNT) first suggestions for test batteries have been emerging (Schmidt et al. 2016; Schultz et al. 2015; Shinde et al. 2016; van Thriel et al. 2012; Zimmer et al. 2014), but further information on the contribution of individual assays is required, and this is also true for reproductive toxicity. In the latter field, reporter assays for nuclear receptors and a Wnt-signalling assay have been combined with a zebrafish embryotoxicity test and the murine embryonic stem cell test within the ReProTect and ChemScreen projects (Kroe et al. 2015; Piersma et al. 2013; Schenk et al. 2010; Sonneveld et al. 2005; van der Burg et al. 2015).

An important step in the further development of test batteries for DNT testing is an evaluation of the technical performance and throughput feasibility of individual assays. A standard approach for this has been the selection of a
small set of compounds, mostly less than ten, that are considered positive and negative controls for the test endpoint chosen. While this is an important first step (Crofton et al. 2011; Leist et al. 2010), relying on this strategy only has made a comparison of the results of cell-based tests difficult. Therefore, further progress depends on the assembly of larger sets of compounds (called a compound library) to be tested in all tests of a battery. Large libraries of several thousand compounds have been established in the Tox21 program (Huang et al. 2016; Richard et al. 2016). Examples for smaller libraries are the 150 compounds of the TG-GATEs project for hepatotoxicity (Grinberg et al. 2014; Igarashi et al. 2015), the 30 compounds of the ESNATS battery (Colaianna et al. 2016; Zimmer et al. 2014) or the set of about 75 compounds assembled by the National Toxicology Program (NTP) of the USA (Pei et al. 2015; Ryan et al. 2016).

Concerning the test endpoints, biochemical measures or reporter gene expression are particularly easy to upscale to high throughput, but the data generated by such tests are difficult to link to classical endpoints of toxicity. The reverse is true for tests of complex and superordinate cell functions such as differentiation, proliferation or migration. Such assays are typically based on laborious cell systems (i.e. long differentiation of cell-based assays) or they require complicated measurement techniques (e.g. electrical activity of neuronal cells) that limit throughput. Nevertheless, it has been repeatedly suggested that such cell function assays will be the first to be used in DNT in vitro batteries (Aschner et al. 2015; Bal-Price et al. 2016; Hirsch et al. 2016; Krug et al. 2016; Stiegler et al. 2011). However, until now the use and inter-laboratory comparison of such assays for screening is still rare. Neurite outgrowth is one of the few examples of a neuronal cell function assay that has been successfully run at high throughput in different tests (Crofton et al. 2012; Hoeling et al. 2016; Krug et al. 2013; Ryan et al. 2016; Stiegler et al. 2011).

Another important function for the development and maintenance of the nervous system is cell migration. For instance, in the developing vertebral central nervous system, neural precursor cells migrate along radial glial cells from the ventricular zone to outer cortical layers (Luhmann et al. 2015). Moreover, throughout life, neural precursors are formed in the subventricular zone, and these then migrate as doublecortin positive neural precursors to their final tissue location (Kaneko et al. 2017). Toxic influences on human central nervous system precursor migration have been studied in the neurosphere migration assay (Fritsche et al. 2005; Moors et al. 2009).

Neural crest cells (NCC) are another migratory cell population arising during early neurodevelopment. We previously established a (low-throughput) assay that measures migration inhibition of neural crest cells (MINC assay) (Zimmer et al. 2012). Initial results of the MINC assay showed that some exemplary compounds specifically affect NCC function (Dreser et al. 2015; Zimmer et al. 2014). Subsequently, a modified version of the assay (cMINC) was adapted for higher throughput by making it experimenter-independent and enabling automated image acquisition (Nyffeler et al. 2016). In the present study, we explored the suitability of the cMINC assay for screening purposes. Moreover, we devised a hit follow-up strategy. In this context, we established and used a panel of secondary assays to obtain further information on the robustness of primary hits and to get an estimate on how confidently the primary hits from a cell function-based screen can be considered as toxicants disturbing a key neurodevelopmental process.

Materials and methods

NCC differentiation

NCCs were differentiated from H9 (WA09; WiCell, Wisconsin) human embryonic stem cells (hESC). The modified line expressed GFP under the control of the Dll1 promoter (H9-Dll1; provided by M. Tomishima, Memorial Sloan Kettering Cancer Centre, New York, NY, USA) as described earlier (Zimmer et al. 2012). Import of the hESC and all experiments utilising them were carried out according to German legislation and under the licence of the Robert-Koch Institute (license number 1710-79-1-4-27).

Differentiation to NCCs was performed exactly as described earlier (Nyffeler et al. 2016; Zimmer et al. 2012). Briefly, the protocol involves differentiation of hESC to rosettes, manual picking of rosettes and FACS-sorting for positive expression of the NCC marker HNK-1 and negative expression of Dll1. The sorted cells were expanded in N2-S medium, consisting of DMEM/F12 (Gibco) and supplemented with 100 µg/ml aptotransferrin, 25 µg/ml insulin, 8.6 mM glucose, 100 µM putrescine, 30 nM selenite, 20 nM progesterone (all purchased from Sigma-Aldrich), 1× GlutaMax and 1% penicillin/streptomycin (both from Gibco), 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml fibroblast growth factor (FGF) 2 (both from R&D). After 27–30 days, cells were cryopreserved until further use. For all experiments, freshly thawed NCCs were used and cultured on poly-l-ornithine/fibronectin/laminin coated plates in N2-S medium supplemented with 20 ng/ml EGF and 20 ng/ml FGF2.

NCC migration setup (‘cMINC’)

The NCC migration assay was performed exactly as described in Nyffeler et al. (2016). Briefly, cells were
seeded (95,000 cells/cm²) in poly-l-ornithine/fibronectin/ laminin coated 96-well plates containing silicon stoppers (Platypus Technologies, Madison, WI, US) to create a circular cell-free area. One day after seeding, stoppers were removed to initiate migration and medium was refreshed. After 24 h, test compounds were added as 5× concentrate (relative to the final test concentration), and after additional 24 h of incubation, migration and viability were measured. A positive control, cytochalasin D (CytoD, 200 nM) was run on every plate for quality control. For standard testing, toxicants were diluted in medium plus dimethyl sulfoxide (DMSO) so that the final DMSO concentration was 0.1% in all wells.

Screen compounds were obtained diluted in DMSO from the NTP as described in Ryan et al. (2016). For the hit-confirmation, all compounds were ordered independently and diluted in DMSO. A list of the test chemicals is given in the Supporting Information. For both the screening and hit-confirmation part, aliquots were stored at −80 °C and for each biological replicate, a fresh aliquot was used. The screening was performed with 4–5 technical replicates, whereas in the hit-confirmation 3–4 technical replicates were done.

Image-based measurement of cell migration and viability

Image acquisition was performed exactly as described in Nyffeler et al. (2016). Briefly, cells were stained with H-33342 and calcein-AM and imaged on a high content imaging microscope (Cellomics ArrayScanVTI, Thermo Fischer). To obtain data on NCC viability, four fields (647 × 647 µm²) outside the migration area were imaged with a 10× lens (approximately 500 cells/field). Viability was defined as the number of H-33342 and calcein double-positive cells as determined by an automated algorithm described earlier (Krug et al. 2013; Stiegler et al. 2011).

To obtain data on cell migration, the centre of the well was imaged in four tiles with the 5× lens. The four images were joined to obtain one micrograph covering an area of 2590 × 2590 µm². For quantification of migration, a software tool (freely accessible at http://invitotox.uni-konstanz.de/) was used to estimate the most likely position of the previously cell-free area, to set thresholds for colour intensity for both dyes, and to count the number of H-33342 and calcein double-positive cells in the region of interest (ROI). The radius of the ROI was reduced to 0.7–0.9 mm to account for the fact that cells could migrate into the cell-free area for 24 h before the treatment was started. The radius of the reduced ROI was chosen in a way that at least 150, but not more than 300 cells, were in the ROI in the untreated condition.

Analysis of migration and viability data

If not mentioned otherwise, viability and migration were normalised to solvent control (0.1% DMSO). Concentration–response curves from averaged data (n = 3 experiments) were fitted using R (R Core Team 2015) and the package ‘drc’ (Ritz and Streibig 2005) to a log-logistic function with four parameters, where the upper asymptote was set to 100%, and appropriate constraints were used for the other parameters (see Fig. S4 and S5). The data points within a curve were weighted with 1/SD; effective concentration (EC) values of the fitted curves were estimated. Efficacy was defined as the amount of migration-inhibition at the EC90 of viability (EC90V). If the EC90V was above the highest tested concentration, the extent of migration at the highest tested concentration was used instead and the greater-than sign was introduced. Specificity was defined as the ratio of EC90V and EC75 of migration (EC75M).

Immunocytochemical characterization

For immunocytochemical staining, cells were seeded on glass as drops (10,000 cells per 10 μl drop) to observe migrating cells at the border. After 2 days in culture, cells were fixed with 4% formaldehyde and 2% sucrose in phosphate-buffered saline (PBS). For intracellular epitopes, cells were permeabilized for 15 min with 0.1% Triton, followed by blocking with 10% foetal bovine serum (FBS) in PBS for 1 h before primary antibodies were applied in 10% FBS in PBS overnight. The next day, cells were washed with 0.05% Tween in PBS and secondary antibodies were applied (1:1000) for 1 h. Cell nuclei were counterstained with 1 μg/ml H-33342 for 10 min. Images were acquired using a point laser scanning confocal microscope Zeiss LSM 700 (Zeiss, Oberkochen, Germany). The list of antibodies used is given in the Supporting Information.

Time-lapse experiment and cell tracking

Cells were seeded under standard cMINC conditions and treated with the test compounds as described in Nyffeler et al. (2016). The DMSO concentration in the medium did not exceed 1%. During the 24-h treatment period, cells were imaged using an Axio Observer.Z1 microscope (Zeiss, Oberkochen, Germany), equipped with an Axiocam MRm camera and an incubation system for constant temperature and CO₂. Phase contrast images of the migration zone were acquired every 10 min using a 5× objective. After the time-lapse, migration and viability were determined as described in the ‘NCC migration setup’ section to ensure that the toxicants worked as under standard cMINC conditions with exposure in a standard incubator.
Cells were manually tracked using the ImageJ plugin, Manual Tracking’ in reverse time. Cells were selected for tracking if they were (1) viable (i.e. not rounded up) at the end of the time-lapse, (2) located in the circular migration zone at the end of the time-lapse and (3) trackable for the entire time-lapse (i.e. did not leave the imaging frame).

For each biological replicate, ten individual cells were tracked per condition (and 20–30 cells for the untreated condition). The cumulated distance of each cell was normalised to the median of the untreated control population. Notably, cells were only tracked if they were viable at the end of the assay. By this way, we purposely selected for ‘healthy’ cells to exclude cell death as a confounding factor. This likely leads to an overestimation of the speed of the cell population. Additionally, only those that were in the migration zone at the end of the assay were tracked. This might result in preferential counting of faster-than-average, mobile cells, which again would overestimate the overall speed. Statistical significance of differences between treated and non-treated conditions was calculated on the combined data of two biological replicates using a Wilcoxon rank sum test. In general, tracking was performed at the EC90V (to ensure that a non-cytotoxic, but rather high concentration was used) except for: LiCl, CdCl2, BPDP (100 µM), taxol (10 nM), ECGG (20 µM), berberine chloride (20 µM), PBDE-99 (20 µM) and valinomycin (1.25 µM). For the remaining 71 compounds, values of logP were obtained. In the process of computing the GRIND2 molecular descriptors, two more compounds had to be removed: saccharin sodium and benzo(b)fluoranthene. Thus, the final series of computing the GRIND2 molecular descriptors, two more compounds had to be removed: saccharin sodium and benzo(b)fluoranthene. Thus, the final series of compounds were obtained from the SMILES provided in the original table, converted to SDFile format using RDKit version 0.9.2 (http://www.rdkit.org) and protonated to pH 7.4 using Moka version 1.1 (Milletti et al. 2007). The values of the molecular weight (MW) and octanol-water distribution coefficient (logP) were obtained using RDKit.

The structures were normalized using standardizer (https://github.com/flaconni/standardiser; accessed: 21st November 2016) and converted to 3D using Corina version 3.494 (Sadowski et al. 1994). These were then used to generate GRIND2 descriptors (Duran et al. 2009; Pastor et al. 2000) making use of Pentacle software version 1.0.6 (http://www.moldiscovery.com/software/pentacle), with default settings. The resulting molecular descriptors were then projected into the principal component analysis (PCA) scores obtained for a collection of 8298 ToxCast and Tox21 compounds (USEPA 2016) characterised using a similar procedure (see Supporting Information Excel File).

From the original 75 library compounds, the following compounds had to be removed because they are salts or contain metallic elements not supported by our method: methylmercury(II) chloride (MeHgCl), acetic acid manganese (2+) salt, bis(tributyltin)oxide, methylcyclpentadienyl manganese tricarbonyl. For the remaining 71 compounds, values of logP and MW were obtained. In the process of computing the GRIND2 molecular descriptors, two more compounds had to be removed: saccharin sodium salt hydrate and benzophenone. Thus, the final series projected in the ToxCast and Tox21 space contains 69 compounds.

Data handling and statistics

If not mentioned otherwise, values are expressed as means ± SD. If not indicated otherwise, experiments were performed at least three times (i.e. using three different cell preparations), with at least three technical replicates per condition.
Results and discussion

Introduction to the biological system

NCCs were differentiated from pluripotent stem cells via a rosette stage, sorted for positive expression of the NCC marker HNK-1, expanded and cryopreserved as described previously (Zimmer et al. 2012). The resulting cells have been extensively characterised for NCC marker expression elsewhere (Nyffeler et al. 2016). We continued this characterisation here by staining for some cell organelles that are relevant for migration or that provide targets for toxicants. This high-resolution imaging showed that NCCs have varying shapes, with the polygonal type being most frequent. As in fibroblasts, a pronounced cortical actin skeleton that extended into cellular processes was conspicuous.

The microtubules stabilised the interior part of the cells, but did not extend into the projections, while nestin, as the typical intermediary filament expressed in these cells, extended throughout the cytoplasm and right into the cell extensions. The few, and quite distinct, mitochondria formed a network around the nucleus, but sometimes extended into protrusions of the leading edge. The Golgi apparatus was always sharply delineated and clearly situated to one side of the nucleus. Its position was often, but not always, in the direction of the leading edge, as observed in some other migratory cells (Fig. 1a).

After this confocal imaging of the cells, conventional, but highly automated fluorescence microscopy was used to obtain high-throughput screening data. Cells were seeded in 96-well plates containing round silicon stoppers, and after one day in culture, the stoppers were removed to allow migration into the circular area (Fig. 1b). After 48 h, the cultures were stained with the viability dye calcein-AM (cytoplasmic localization in live cells) and with Hoechst 33342 to visualise the nuclei of all cells. The number of viable cells (defined as the number of calcein-positive nuclei), and the number of migrated cells were determined using previously published algorithms (Krug et al. 2013; Nyffeler et al. 2016; Stiegler et al. 2011).

Using this procedure, several published endpoint-specific controls (Aschner et al. 2016; Barenys et al. 2016; Zimmer et al. 2012) were tested for their effect on cell migration. The JNK inhibitor SP600125 and the Src-kinase inhibitor PP2 inhibited migration in a concentration-dependent manner at non-cytotoxic concentrations in the micro-molar range. As expected, also, the Gai-signalling inhibitor pertussis toxin, as well as the polyphenol EGCG (preventing laminin from binding to the integrin β1 receptor) inhibited NCC migration (Fig. 1c). All these largely different positive controls yielded highly reproducible concentration-dependent inhibition data in three separate experiments and allowed to exemplify effect quantification according to our earlier developed preliminary prediction model for the cMINC test. We therefore decided that the assay was ready for a screen of multiple unknown compounds, and we adopted our earlier definition of compound efficacy as ‘the extent of migration inhibition at the highest non-cytotoxic test concentration (EC90V)’ and of compound specificity as ‘the ratio of the EC90V and the concentration inhibiting migration by 25% (EC75M)’.

Overview of the testing strategy

The library to be tested has been assembled at the NTP of the USA to probe the feasibility and performance of screening approaches in the fields of neurotoxicity and developmental neurotoxicity (Pei et al. 2015; Ryan et al. 2016). It contains 75 unique compounds, five of which (saccharin, deltamethrin, triphenyl phosphate, MeHgCl and phenobarbital) were included as duplicates for internal consistency control; it is thus short-named as ‘NTP80-list’ here. The library contained some known (developmental) neurotoxic compounds of the group of pesticides, drug-like compounds and industrial chemicals, in addition to large sets of polycyclic aromatic hydrocarbons (PAH) and flame retardants (FR) (Fig. S1). Moreover, five negative controls were included (acetylsalicylic acid, acetaminophen, n-glucitol, t-ascorbic acid and saccharin sodium salt). The library was delivered in the form of 20 mM stocks in DMSO, except for few compounds of lower solubility.

As the production of NCC is cost- and time-demanding, a tiered screening strategy was chosen (Fig. 2). In a first step, all compounds were tested at the technically highest concentration (1:1000 of the stock). If cytotoxicity was observed (viability <85%), the compound was retested at progressively twofold dilutions, until a non-cytotoxic concentration (NOAELv) was determined. Note that in this pre-screen, we deliberately chose a non-stringent viability threshold (normally, a cutoff of 90% is used) to include a high percentage of potentially specific toxicants. At the NOAELv, migration was measured in three independent experiments. Compounds were classified as ‘negative’ if average migration was ≥90% (again, the threshold for migration-inhibition was chosen less stringent than in the standard procedure). All other compounds were considered as ‘potential positive hits’ and underwent hit confirmation testing in the second tier. For this purpose, all relevant compounds were purchased from a different source (confirmation of compound identity and integrity), and re-tested in the migration assay. At this stage, six dilutions were tested simultaneously. A compound was classified as ‘positive
Fig. 1 Introduction to the biological system. a Cryopreserved NCCs were thawed and plated on coverslips. After 2 days, they were fixed, and various cellular structures and markers were stained; H-33342 (depicted in blue) was used to visualise the nuclei. NCCs migrating into cell-free zones were imaged using a confocal microscope. The scale bar corresponds to 25 µm. p75 NCC marker; Nestin stem cell marker; TOM20 mitochondrial marker; Giantin golgi marker. b Graphical representation of the assay scheme: cells were seeded in 96-well plates containing silicone stoppers to create a cell-free circular area. After 1 day, the stoppers were removed, and cells were allowed to migrate for 24 h before the test compounds were added for additional 24 h. For imaging with an automated screening microscope, cells were visualised using H-33342 (intercalates into DNA) and calcein-AM (fluorescent in viable cells only). Imaging of the central zone allowed the quantification of cell migration; the cell viability was determined from images of the well periphery. c Examples for endpoint-specific controls. The known biological activities of the test compounds are indicated on top of the diagrams in blue. The light grey dotted line indicates the 100% value. The other two grey lines are drawn at 90 and 75% to indicate thresholds for reduced viability and migration, respectively. All values are normalised to untreated controls. Data are mean ± SD from three experiments. In the microscopy images, the viable cells are displayed in green and nuclei of viable cells in red (colour figure online).
hit' if migration was <75% at EC90V. In the third tier of this study, these 'positive hits' were further studied: (1) the cMINC assay was repeated under conditions that prevent proliferation; (2) a transwell migration assay was used to test whether a different migration assay would confirm the result; (3) cells were traced to assess cell speed as a different endpoint. This final tier was included to obtain information in how far technically-defined ‘screen hits’ can indeed be considered as compounds associated with the toxicological hazard of ‘interfering with the fundamental neurodevelopmental process’ of NCC migration (Fig. 2).

Results of the screening

During the screening of the NTP80-list, only 12 compounds proved to be cytotoxic at the highest tested concentration. For these substances, a NOAEL was determined, before migration data were obtained. For all other compounds, migration was measured at the highest possible concentration (usually 20 µM). On each plate, Cytod was run as a positive control and reduced migration to 30–70% compared to solvent control (Fig. S2). There was only limited variation among assays and cell lots, indicating that the assay worked reproducibly.

In tier 1, out of the 80 compounds, 27 ‘potential positive hits’ were identified (Fig. 3), and none of the compounds triggered an increase in migration. For one compound (benzo[b]fluoranthene, the standard quantification procedure was not possible, as the fluorescent compound interfered with image acquisition of the H-33342 channel. However, manual inspection of the calcine-stained cells did not indicate any inhibition of migration, and hence no further experiments were conducted.

Among the ‘potential positive hits’, there were ten of 12 tested FR, seven of 17 pesticides, six of the 15 drug-like compounds as well as two PAHs and one industrial chemical. None of the five negative controls inhibited migration.

Hit confirmation with primary assay

In tier 2, new stocks were prepared for all ‘potential positive hit’ compounds, and full concentration–response
### 3.3 Results and discussion

**Fig. 3** Results of the screening. Compounds were screened according to the procedure indicated in Fig. 2 at a 1:1000 dilution (mostly 20 µM) if they were not cytotoxic at this concentration. Otherwise, they were tested at the highest non-cytotoxic concentration. Compounds are ordered according to the screen results from most migration-inhibiting (top) to least migration-inhibiting. For each compound, viability (grey diamonds) and migration (blue circles) at the highest tested non-cytotoxic concentration are displayed. The vertical lines are at 80, 85, 100, 120% of control and indicate the thresholds for viability (grey) and migration (blue). Compounds reducing migration by ≥20% underwent subsequent hit confirmation testing. For one compound (benzo[bf]fluoranthene), migration could not be evaluated due to interference of the chemical with the fluorophores of the test. The chemical classes of the compounds were FR flame retardant, PAH polycyclic aromatic hydrocarbon, ind industrial chemical, pest pesticide, drug drug-like compound, neg negative control. On the left side of the overview, the NOAEL of viability is displayed in a white box for compounds not cytotoxic at the highest tested concentration (i.e. the NOAEL is greater than the given value) and in a grey box for all other compounds (colour figure online).
curves were obtained. Both, the viability and migration data, were fitted to a log-logistic function (Fig. S4 and S5) and specificity and efficacy were calculated as described in Nyffeler et al. (2016).

Of the 26 'potential positive hits' (MeHgCl was twice in the library), 23 were confirmed (Figs. 4, 5, S3A). The three non-confirmed compounds were the two PAHs acenaphthylene and dibenz[a,c]anthracene as well as bisphenol A (Fig. S3B). At this stage, all 23 confirmed hits fell into the chemical classes of FR (10 hits), pesticides (7 hits) and drug-like compounds (6 hits).

The hits belonging to the FR group showed a rather large specificity (Fig. 4), and a potency in the low (single-digit) micromolar range. Tetrabromobisphenol A (TB-BPA) was the most potent FR, followed by the organophosphorus compounds and polybrominated diphenyl ethers (PBDEs). The six hits amongst the eight organophosphorus FR have in common that they contain at least one aromatic side chain, whereas the two non-hits did not show this feature. For PBDEs, it seems that an increasing number of bromine atoms reduces the effect, as PBDE-47 (4 bromines) was more effective than PBDE-99 and PBDE-153 (5 and 6 bromines, respectively).

Of 15 tested drug-like compounds, six were found to be migration-inhibiting after the hit confirmation phase (Fig. 5a; Fig. S3A). Colchicine, previously known as a NCC migration-inhibitor (Nyffeler et al. 2016), was the most potent hit (low nanomolar range). Hexachlorophene, a chlorinated compound used as a disinfectant, and diethylstilbestrol (previously used as a contraceptive) were amongst the hits. For valinomycin, the hit confirmation showed a non-orthodox and biphasic concentration–response behaviour (confirmed in altogether six experiments). In the low nanomolar range, the cell viability dropped reproducibly by about 10%, but was not affected by increasing concentrations up to the micromolar range. At concentrations of about 2–4 µM, a pronounced effect on migration was measured. To display the effect of the compound in the micromolar range in a way comparable to other substances, the data were normalised to the data obtained in the lower nM range instead of the usual comparison to solvent controls. Amongst the drug-like compounds, also berberine chloride and 6-hydroxydopamine were confirmed as migration-inhibitors, but they had a rather low specificity and efficacy (Fig. S3A).

Seven out of 17 tested pesticides inhibited NCC migration (Fig. 5b; Fig. S3A). MeHgCl and rotenone were confirmed as known migration-inhibitors (Dreser et al. 2015; Moors et al. 2009; Zimmer et al. 2012), while pyrethroids had no effect at all. Concerning a role of choline esterase inhibition, carbaryl (Fig. S3A) was the only positive hit of the carbamate group (n = 3) and it had a low efficacy (29%). Chlorpyrifos showed specific effects at relatively high concentrations of 10–20 µM. The three organochlorine pesticides DDT, dieldrin and heptachlor were among the most efficient compounds in the library, although
concentrations of several micromolar were required to trigger specific effects. In the case of DDT, we followed up on the screen finding by examining effects of the two congeners 2,4′-DDT (op-DDT; 1,1,1-Trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane) and 4,4′-DDT (pp-DDT; 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane). The initial finding of a highly efficient inhibition of migration was confirmed with both compounds (Fig. 5c).

In summary, most of the screen hits were confirmed, and additional information on potency and efficacy could be obtained (Fig. S7 and Table S1). The cMINC screen detected compounds with a potency range over three orders of magnitude (Fig. 6a), and a higher dynamic range may still be obtained using higher start concentrations for the hit finding. The yet little-characterised group of FR had the most consistent effect on NCC migration, whereas all PAHs and industrial chemicals tested were found to be negative.

Representation of chemical space by the library and comparison of hits to published data

As the screen hits were unevenly distributed amongst the groups of compounds assembled in the NTP80-list, it was interesting to see whether some physicochemical properties of chemicals were particularly overrepresented amongst the hits. On the first view, it appeared as if lipophilic compounds (e.g. organochlorines or FR with aromatic substituents) may be particularly prone to be hits in the cMINC. To test this hypothesis, a systematic cheminformatics approach was used. First, we investigated whether the hits and non-hits significantly differ in their MW, logP, number of rotatable bonds (NRB), number of hydrogen bond acceptors (NHBA) and polar surface (TPSA) using a Wilcoxon rank sum test. The group of hits had significantly higher MW (p < 10\(^{-6}\)), logP and NRB (p < 0.01) (data not displayed).

Also, some parameter combinations (e.g. logP AND MW) differed significantly between hits and non-hits, but none of these combinations was able to specifically predict a hit compound.

Representation of all library compounds in the MW–logP space (Fig. 6b) illustrated that both properties were weakly correlated (r = 0.51), while MW and logP were largely independent (r = 0.2) for the screen hits. To obtain a less biased overview of compound properties, grid-independent descriptors (Duran et al., 2009; Pastor et al., 2000) (GRIND2) were computed as described in the “Methods” section. In order to anchor the display of the NTP80-list according to these descriptors, the same parameters were also obtained for a set of ToxCast and Tox21 compounds (n = 8298). Then, two principal components (PC1, PC2) were obtained for this large chemical group using PCA, and the NTP80-list was projected in this principal component space (Fig. 6c). The hits obtained in our screen

Fig. 5 Hit confirmation with the primary assay: drug-like compounds and pesticides. a, b Compounds identified as potential hits in the screen were re-ordered and concentration-response curves were obtained for viability (grey triangles) and migration (blue circles). All values are normalised to the solvent control (0.1% DMSO). The horizontal light grey dotted line indicates the 100% value for easier reading of the diagrams. The other two grey lines are drawn at 90 and 75% to indicate the threshold for reduced viability and migration, respectively. A log-logistic function with constraints was fitted to the concentration-response curve and the EC90 of viability and the EC75 of migration were interpolated. The ratio between these two values was termed ‘specificity’, whereas ‘efficacy’ was defined as the amount of migration-inhibition at the EC90 of viability. To compare the results with other compounds, the data of valinomycin were normalised to 100% (i.e. multiplied with 100/89). Data are mean ± SD from three experiments. c Two isomers of dichlorodiphenyltrichloroethane (DDT) and the commercial DDT mixture were retested in the cMINC assay (5 µM). Data are mean ± SD from three experiments. op-DDT 2,4′-DDT, pp-DDT 4,4′-DDT

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occupied a large area of this chemical space, i.e. they were unlikely to reflect only a very narrow chemical subgroup. In a next step, a large number of classification models were built from the screen hits, and they were validated by leave-one-out procedures. However, a convincing separation of hits and non-hits could not be obtained, based on the use of these chemical descriptors (not shown), i.e. cMINC toxicity was not predictable based on the structural information available.

For further information on the cMINC hits, they were compared to available data from a similar screen for neurite outgrowth of induced pluripotent stem cell-derived neurons and to screen data obtained within the Tox21 program (Ryan et al. 2016). In general, the potency ranking of compounds correlated well between cMINC and the ‘Ryan assay’, but the apparent toxicant potencies were mostly three- to tenfold higher for the migration assay (Fig. 6d). The cMINC also appeared to be more sensitive than the majority of Tox21 assays, at least for the hit compounds identified in the migration assay. In fact, for most compounds that specifically blocked NCC migration, the cMINC was the most sensitive of all the tests that were compared. Amongst these particularly potent migration inhibitors were rotenone, hexachlorophene, DDT,
PBDE-47, BPDP, heptachlor, EHDP and dieldrin. In contrast to this, hits like PBDE-99, PBDE-153 and berberine had EC75M values in the cMINC that fully overlapped with the range of concentrations that affected most Tox21 assays.

Altogether, the hits identified here were generally bioactive in other tests. Concerning the prioritisation and follow-up of such compounds, the cMINC appeared to be particularly sensitive for certain toxicants, and such toxicants came from a broad range of structural templates, not easily covered by a quantitative structure-activity relationship (QSAR) approach.

**Cell tracking as follow-up assay**

After the comparison of the cMINC hits to data in the literature, we tested in a second step the biological consistency of screen hits. For this purpose, we examined in how far the inhibition of migration of NCC was confirmed, when other (secondary) tests for the same endpoint were used.

The first secondary assay assessed the migration speed of single cells. The test conditions were identical to the cMINC, but the analytical endpoint was time-lapse imaging of the cells. The average cell speed (expressed as cumulated travelled distance within 24 h) was determined from the tracks of the cells on the dish (Nyffeler et al. 2016). Untreated cells typically covered a distance of about 1000 µm in 24 h. Cells treated with the exemplary migration toxicant LiCl covered only about half the distance (Fig. 7a). All six positive controls of this test, including the recently characterised migration-inhibitor EGCG (Barenys et al. 2016) were used at non-cytotoxic concentrations, and they reduced the speed by 20–45% (Fig. 7b).

The 'cMINC hits’ were also tested at their EC90V (or at the highest non-cytotoxic concentration reached). To reduce the workload, only two exemplary FR were tested: BPDP was chosen as representative for the group

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**Fig. 7** Cell tracking as follow-up assay. Cells (plated at standard cMINC conditions) were imaged during the 24-h treatment period to assess the cumulated travelled distance of individual cells by manual tracking. a Visualisation of cell tracks for untreated cells and cells treated with 20 mM LiCl. b Establishment of the cell tracking assay using endpoint-specific controls and compounds known to inhibit NCC migration in the cMINC assay. For each treatment condition, 20 individual cells were tracked and the cumulated travelled distance was normalised to the untreated control. The box represents the first and third quartiles, the black line the median (number given in the rectangle) and the whiskers are at an interquartile range of 1.5. c Performance of the screen hits in the cell tracking assay: green flame retardants; blue pesticides; pink drug-like compounds. Data are from two independent biological experiments, i.e. the box represents data from 20 tracked cells. Statistical significance of differences between treated and non-treated conditions was calculated using a Wilcoxon rank sum test. Statistical indicators are given below the boxes *p < 0.05, **p < 0.01, ***p < 0.001. Names of screen compounds that reduced the speed of migration are bold. EGCG epigallocatechin gallate, RA retinoic acid, TB-BPA 3,3',5,5'-tetrabromobisphenol A, 6-OHDA 6-hydroxydopamine hydrochloride, DES diethylstilbestrol, 6PH hexachlorophene (colour figure online)
of organophosphorus FR and PBDE-47 for the group of PBDEs. The test revealed that only four of the cMINC hits reduced cell speed to a similar extent as the positive controls (by ≥20%): DDT, heptachlor, carbaryl and hexachlorophene (Fig. 7c). For these toxicants, a reduction of cell speed appears to be a major mechanism responsible for reduced migration. Compounds like TB-BPA, rotenone or valinomycin did not affect cell speed at all. For such toxicants, the effect measured on a population level in the cMINC is likely caused by a change of the percentage of migratory cells, by altered directionality behaviour or by indirect effects that would need further investigation. For a large group of compounds, the reduction of speed was small, but statistically significant. It is not clear at present whether such relatively minor effects are of biological significance. More elaboration on the underlying mechanisms, extensive testing of additional positive and negative controls to establish a robust prediction model and more refined statistics would be required to come to a clearer conclusion on the impact that toxicants have on NCC migration speed.

However, also the presently available data set clearly indicated that the choice of the analytical endpoints can lead to different conclusions (hit subsets) in chemical screens. Moreover, it became clear that one key neurodevelopmental process, such as NCC migration, has a complex underlying biology that may be only partially captured by any given single test. In the case examined here, there were, for instance, compounds that reduced single-cell migration speed, while others did not. Compounds from both groups reduced the number of cells found in the circular area of the cMINC and had thus been classified as migration-inhibitors.

Re-testing of confirmed hits under conditions that prevent proliferation

Differences between the cMINC and the cell tracking assay may be re-considered if compounds not affecting the migration speed could be shown to inhibit cell proliferation, and thus to be false positives in the cMINC. Therefore, the cMINC assay was repeated for all confirmed hits under conditions that do not allow any NCC proliferation (1 μM cytosine arabinoside, AraC, (Nyffeler et al. 2016). All toxicants were tested at the EC90V (same concentration as for cell tracking), and a similar threshold for impairment of function (i.e. 20% reduction) was set. A full prediction model has not been developed for the cMINC in the presence of AraC, but for the purpose of this comparison, we considered it plausible that a reduction of cells in the migration area to 80% or below can be considered (1) biologically relevant, and (2) comparable to the tracking assay. Most of the compounds were confirmed to be migration-inhibitors under these conditions (Fig. 5f). Thus, the number of artefacts induced by effects on proliferation in the standard cMINC was low, and the data in the absence and presence of AraC were well-correlated (Fig. 5f). Only TPP, PBDE-153, MeHgCl, berberine chloride and carbaryl were not confirmed as hits. This puts a direct effect of these compounds on NCC migration at question. In order to measure the impact of chemicals on migration, independent of proliferation, and in the absence of an additional toxicant (AraC), a short-term migration test was established.

Re-testing in a short-term transwell migration assay

Migration through a membrane, along a chemotactic gradient can be tested within few hours, and is thus little influenced by proliferation in a cell population. Therefore, a transwell migration assay was established. For this purpose, cells were seeded in inserts (upper compartment of a two-compartment dish). FBS was added to the lower compartment to stimulate the cells to migrate into the direction of this chemotaxant. Under these conditions, control NCC translocated through the porous membrane into the lower compartment (Fig. 8a). After 6 h, migrated cells were fixed, stained, imaged and manually counted. In a typical experiment, stimulation with 5% FBS led to the migration of 100 cells/image field, while <3 cells migrated in the absence of FBS (Fig. 8b). The endpoint-specific controls CytoD, taxol and EGCG reduced transwell migration by >50%, while several known unspecific toxicants (Nyffeler et al. 2016) reduced transwell migration by ≤25% (Fig. 8c). Based on these data, the threshold for migration-inhibition was set at 25% inhibition of migration. As next step, five well-established cMINC toxicants (Nyffeler et al. 2016) were tested at their EC90V or a corresponding reasonable test concentration. LiCl strongly (>50%) inhibited transwell migration, As2O3, CdCl2 and acrylamide had a moderate effect (25–50% range). Only retinoic acid (albeit a strong migration-inhibitor in the cMINC assay) did not specifically affect transwell migration (<25%). Thus, this small evaluation of the assay showed that the hits largely overlapped with the cMINC, but that some toxicants showed clear differences. One potential reason may be that the assays measure short-term vs long-term consequences of chemical exposure, which may be particularly important for slow-acting nuclear receptor ligands, such as retinoic acid. Another conceptual difference of the assays is the distance of migration required to lead to a positive effect. In the transwell assay, mainly the ‘mobility’ of a cell is assessed. Cells either translocate across the very thin membrane (at any
time point of the 6-h assay window), or they remain in the upper compartment. Thus, we hypothesise that compounds that slow down cells rather than immobilise them would not reduce transwell migration.

Eventually, all cMINC hits were tested at their EC90V (or highest available concentration), and all compounds reduced transwell-migration by 30–45% (Fig. 8d). Notably, also compounds with small effects in the cMINC assay like berberine chloride, PBDE-99 and MeHgCl had a robust effect in the transwell assay.

In summary, all hits from the cMINC were confirmed in this third follow-up migration assay, and in general, it is likely that most cMINC hits from this screen do indeed specifically affect NCC migration.

Conclusions and outlook

The screen of the NTP80-list of 75 compounds for inhibition of migration of NCCs yielded 26 initial screen hits, 23 of which were confirmed and quantified as to their efficacy, potency and specificity. All hits fell into the classes of FR, pesticides or drug-like compounds, whereas all PAHs and industrial chemicals were negative. All of the confirmed hits in our screen (with the exception of TB-BPA) were among the active compounds identified in a neurite-outgrowth screen (Ryan et al. 2016), and five of the six compounds affecting neurites selectively were also among the hits that specifically affected NCC migration.

Fig. 8 Transwell migration assay as follow-up assay. a Schematic indicating the assay principle: cells were plated into transwell inserts and stimulated to migrate with 5% foetal bovine serum (FBS) in the lower compartment. Test items were added to both compartments. After 6 h, cells were fixed, stained and the number of cells that migrated through the transwell membrane were manually counted. b Exemplary microscopy image of migrated cells (purple blotches) on the lower surface of the membrane. The scale bar corresponds to 150 µm. c Benchmarking of the transwell assay with endpoint-specific controls (dark grey), unspecific compounds (white) and compounds well established to inhibit NCC migration in the cMINC assay (light grey). Transwell migration is measured as the number of migrated cells relative to cells stimulated with 5% FBS alone. The dotted grey line at 75% separates unspecific compounds from endpoint-specific controls. Data are expressed as mean ± SD from at least two independent biological experiments. d Performance of the screen hits in the transwell migration assay: green flame retardants; blue pesticides; pink drug-like compounds. Data are expressed as mean ± SD from three independent biological experiments. FBS foetal bovine serum, CytoD cytochalasin D, EGCG epigallocatechin gallate, HCTL l-homocysteine thiolactone, RA retinoic acid, TB-BPA 3,3′,5,5′-tetrabromobisphenol A, 6-OHDA 6-hydroxydopamine hydrochloride, DES diethylstilbestrol (colour figure online)
The potential developmental neurotoxicants were then investigated in three follow-up tests, and all compounds were positive in at least one additional assay (Fig. 9a). A comparison of the hits showed that the two organochlorines, DDT and heptachlor, had the strongest effects on NCC migration, albeit low micromolar concentrations were required for this manifestation of toxicity. Comparison of hits across multiple other published assays indicated that NCCs seem to be particularly sensitive to hexachlorophene and diethylstilbestrol as compared to other cell types or biological processes (Fig. 6d). The chemical groups with the highest hit rate were the organophosphates FR. Our data suggest that such compounds, if foetal concentrations >1 µM are reached, might disturb development similar to the PBDE FR that are being phased out from the markets because of their hazard (Linares et al. 2015). In this context, it is noteworthy that novel templates for FR have been recently characterised and that these were considerably less toxic to NCCs (Hirsch et al. 2016).

An important question raised by our study is, “why do different migration assays yield different hits?”. We favour the explanation that (1) each ‘fundamental neurodevelopmental process’ is a complex assembly of cellular functions; (2) it combines multiple adverse outcome pathways (AOPs) and can, therefore, be affected by many chemicals in different ways; and (3) each test method focuses on different aspects within a fundamental neurodevelopmental process and, therefore, reflects key events of different AOPs. Some empirical evidence suggests that this hypothesis holds true in many cases: for instance, ‘neurite outgrowth’ could actually mean in a given test: dendrite growth, axon growth, axon branching, growth cone extension, etc. It could also be the extension of already well-established neurites, or rather be the initiation of neurite formation; or it could refer to peripheral neurons or central neurons. There is clear evidence that such processes may be affected differentially (Hoelting et al. 2016; Kim et al. 2002). A similar heterogeneity holds true for neuronal network activity (e.g. type of neurons used; presence or absence of glia), and also for migration (Fig. 9b).

Different migration assays are complementary or partially overlapping, but never identical. Apart from the

The explanation that (1) each ‘fundamental neurodevelopmental process’ is a complex assembly of cellular functions; (2) it combines multiple adverse outcome pathways (AOPs) and can, therefore, be affected by many chemicals in different ways; and (3) each test method focuses on different aspects within a fundamental neurodevelopmental process and, therefore, reflects key events of different AOPs. Some empirical evidence suggests that this hypothesis holds true in many cases: for instance, ‘neurite outgrowth’ could actually mean in a given test: dendrite growth, axon growth, axon branching, growth cone extension, etc. It could also be the extension of already well-established neurites, or rather be the initiation of neurite formation; or it could refer to peripheral neurons or central neurons. There is clear evidence that such processes may be affected differentially (Hoelting et al. 2016; Kim et al. 2002). A similar heterogeneity holds true for neuronal network activity (e.g. type of neurons used; presence or absence of glia), and also for migration (Fig. 9b).

Different migration assays are complementary or partially overlapping, but never identical. Apart from the
above arguments, identity is also not possible, because each experimental system and test protocol is prone to different sets of artefacts, false positives and false negatives. For instance, the cMINC not only measures migration, but the test endpoint may also be affected by proliferation, cell death, and signalling disturbances (Fig. 9b, top row—blue box). The contributions of various biological processes are different for each migration assay. Moreover, migration itself is a highly complex process, involving several cell biological functions (Fig. 9b, bottom row). In the cMINC assay, migration is measured as the number of cells in the migration zone at the end of the assay. A reduction can be caused by several mechanisms and the assay does not provide information about which subfunction was affected. Successful migration requires that cells adhere to the substrate, but that they can also sever bonds to the extracellular matrix (adhesion). A cell also needs to be able to remodel the cytoskeleton and cytoplasmic structure in the right way (motility). Moreover, cell migration normally is a cyclic sequence of moving phases and rest phases, and the ratio of these phases is described by ‘continuity’. Finally, cells can change direction randomly, or rather keep an overall direction (directuality) and this has in the end a large effect on the overall net distance covered.

The four assays used here measure different sets of the above migration features. The cell tracking assay captures ‘distance’ and ‘continuity’ but excludes cell proliferation and cell death. The transwell assay captures ‘motility’ and ‘adhesion’. As the cells need to react to the chemoattractant stimulus, also some aspects of cell signalling, (‘polarity’ and ‘sensing’) are important, whereas the sub-functions that dominate the cell tracking assay are negligible. As a confounding factor, cell death might also be involved in the transwell assay, whereas proliferation effects are negligible due to the short assay time. The assays presented in this study, therefore, cover different aspects of NCC migration and NCC function. They provide thus complementary information. A single compound may have several simultaneous effects on cells, e.g. change adhesion, change cell stiffness, change cell speed and hence have an effect in several assays, but not necessarily in all. This explains the differential activity pattern observed here (Fig. 9a). With respect to a test strategy, we conclude from this that it is dangerous to rely on a single test if a big and complex biological process like cell migration is investigated. For practical purposes, we suggest to use the cMINC as primary screening assay, and to characterise the hits with at least one further (better more) secondary assay.

In order to assemble more and more information on screen hits, three forms of follow-up need to be considered, and some examples for them have been provided in our study: (1) technical follow-up (confirmation of findings in the same assay): in the present study, 23 of 26 screen hits were confirmed in the hit-confirmation phase. This high level of confirmation (88%) indicates that the assay is technically robust, but not perfect; (2) biological consistency (confirmation of findings in another assay for the same endpoint): the primary hits were tested in three follow-up assays, and the transwell assay confirmed findings from the cMINC. The other assays showed a more heterogeneous picture. As a general strategy, a given test could be combined with other cell function assays (Hirsch et al. 2016), with biochemical assays (Ryan et al. 2016), with tests on simple model organisms (Behl et al. 2016) or with transcriptome analysis (Pallocca et al. 2016) to provide more biological information; (3) mechanistic consistency amongst related compounds: for drug discovery screens it is common to look for structurally related compounds that give consistent hit patterns. For hit singletons, the likelihood of artefacts and wrong conclusions is known to be high. For toxicological screens, there are usually not enough compounds for direct QSAR or read-across approaches, but corresponding compounds may be selected for a follow-up. In the present study, all PBDEs were among the hits and all organophosphorus FR with aromatic side chains, whereas aliphatic ones were negative. Thus, for such compounds, there is higher confidence that this type of basic structure is linked to a hazard. For the organochlorines, there were clearly positive and negative compounds (for example, lindane was negative). Clear consistency has been shown here for DDT congeners that showed comparable efficacy.

Another form of mechanistic consistency testing involves exploration of a suggested mode of action. For instance, identification of chlorpyriphos as a hit would suggest that acetylcholine esterase may be a target. This could be tested by examination of other (structurally unrelated) choline esterase inhibitors. Alternatively, chlorpyriphos oxone may be tested and should be more potent if the common target was choline esterase. Similar considerations would apply to the follow-up of parathion as a hit by testing of paraaxon, or to the consideration whether the oestrogen receptor plays a role in the toxicity of diethylstilbestrol. One may follow up with several estrogenic compounds, but the high concentration of diethylstilbestrol required to inhibit NCC migration already suggests now that this effect is independent of oestrogen receptor activation.

In summary, our study has provided rich information on issues and potential solutions associated with the use of cell function assays for screening. For the future, we would like to pinpoint some important areas of work. For instance, a hit confirmation rate of 80% may sound good. However, if five such assays with 20% false positives are combined, the overall false positive rate (probability of a compound being a hit in at least one of the five assays) would be 67%. How will the field deal with this? One approach could be
to combine tests in biological networks guided by molecular mechanisms/AOPs (Jaworska et al. 2015; Juberg et al. 2016), or to use biological information across assays in form of a biological read across (Hartung 2016; Patlewicz and Fitzpatrick 2016; Strickland et al. 2016a, b). Another old, but continuously present and topical issue (Judson et al. 2016; Leist et al. 2010) is the problem of cytotoxicity. The definition of hit specificity is often arbitrary, and more experience needs to be collected for each test to differentiate real hits from those that only reflect cytotoxicity. To advance on all these questions, it will be most important to run the same set of compounds described here in as many tests as possible, or to take similar approaches with other compound sets.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.
4 Results. Manuscript 3

A structure–activity relationship linking non-planar PCBs to functional deficits of neural crest cells: new roles for connexins

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Abstract

Migration of neural crest cells (NCC) is a fundamental developmental process, and test methods to identify interfering toxicants have been developed. By examining cell function endpoints, as in the ‘migration-inhibition of NCC (cMINC)’ assay, a large number of toxicity mechanisms and protein targets can be covered. However, the key events that lead to the adverse effects of a given chemical or group of related compounds are hard to elucidate. To address this issue, we explored here, whether the establishment of two overlapping structure–activity relationships (SAR)—linking chemical structure on the one hand to a phenotypic test outcome, and on the other hand to a mechanistic endpoint—was useful as strategy to identify relevant toxicity mechanisms. For this purpose, we chose polychlorinated biphenyls (PCB) as a large group of related, but still toxico logically and physicochemically diverse structures. We obtained concentration-dependent data for 26 PCBs in the cMINC assay. Moreover, the test chemicals were evaluated by a new high-content imaging method for their effect on cellular re-distribution of connexin43 and for their capacity to inhibit gap junctions. Non-planar PCBs inhibited NCC migration. The potency (1–10 µM) correlated with the number of ortho-chlorine substituents; non-ortho-chloro (planar) PCBs were non-toxic. The toxicity to NCC partially correlated with gap junction inhibition, while it fully correlated (p < 0.0004) with connexin43 cellular re-distribution. Thus, our double-SAR strategy revealed a mechanistic step tightly linked to NCC toxicity of PCBs. Connexin43 patterns in NCC may be explored as a new endpoint relevant to developmental toxicity screening.

Keywords Cell migration · Cell tracking · Cytotoxicity · High-content imaging · Developmental toxicity · Human stem cells

Abbreviations

AhR Aryl hydrocarbon receptor  
AP1 Activator protein 1  
AR Androgen receptor  
CAR Constitutive androstane receptor  
cMINC Circular MINC  
Cx43 Connexin43  
Cx43pq Connexin43 plaques  
CytoD Cytochalasin D  
DMSO Dimethyl sulfoxide  
EC Effective concentration

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Introduction

Polychlorinated biphenyls (PCB) are environmental contaminants and are developmentally neurotoxic. Prenatal exposure to PCBs has been associated with poorer short-term memory and verbal comprehension as well as a lower IQ (Jacobson and Jacobson 1990). Mechanisms of PCB neurotoxicity are not fully understood, but effects on neurotransmitters, hormonal balance and intracellular signaling are discussed (reviewed in Kodavanti 2005; Westerink et al. 2013).

We previously reported that PCBs are toxic to a particular fetal cell type, the neural crest cell (NCC). Such NCC arises during early neurodevelopment at the border of the neural plate. After delamination, they migrate to their target site in the body and give rise to a variety of neural and non-neural cell types. By screening diverse environmental contaminants, we found that also several PCBs were among the chemicals that disturb NCC migration at non-cytotoxic concentrations (Dreser et al. 2015; Nyyfèrèt al. 2017b; Zimmer et al. 2014). However, at present it is unclear which PCB congeners exert this toxicity and by which mechanism migration-inhibition occurs.

The structure of PCBs consists of a biphenyl skeleton with 1–10 chlorine substitutions, leading to 209 different PCB congeners. They have low electrical, but high thermal conductivity. Therefore, PCBs have been used as dielectric and heat transfer fluids in electrical equipment. They are environmentally stable and due to their high lipophilicity tend to accumulate along the food chain (Henry and DeVito 2003; World Health Organization 2000).

Although they share important structural features, PCB congeners differ largely in basic toxicokinetic and physico-chemical properties like lipophilicity and volatility. In general, higher chlorinated congeners are less absorbable, but also less readily metabolized and excreted, which leads to their accumulation, particularly in tissues with high lipid contents (Henry and DeVito 2003; World Health Organization 2000).

Moreover, they have different affinities for several cellular receptors. For example, non-ortho chlorinated PCBs tend to be planar, and they share structural similarity with dioxins. Hence, several of them can activate the dioxin receptor (aryl hydrocarbon receptor, AhR) (Safe 1994). Conversely, ortho-chlorinated, non-planar PCBs have various other cellular targets, such as ryanodine receptors (RyR) and several nuclear receptors (Al-Salman and Plant 2004). In addition, other cellular functions have been shown to be impaired by PCBs, for example, dopamine uptake and binding of dopamine to its receptor (Mariusson et al. 2001; Wigestrand et al. 2013).

Several groups have reported that PCBs inhibit gap junction intercellular communication (GJIC) and dislocalize the corresponding proteins in several cell types (Bager et al. 1994, 1997a, b; Brevini et al. 2004; Hamers et al. 2011; Hemming et al. 1991; Kato et al. 1996; Machala et al. 2003; Simeckova et al. 2009). Gap junctions (GJ) are intercellular channels consisting of conniving oligomers. Each cell forms a connexon consisting of six connexin proteins. Upon connection of two connexons from two neighboring cells, a GJ is formed and small molecules up to 1.2 kDa can be exchanged (Schalper et al. 2014).

Most cells express several connexins, and the connexin pattern differs profoundly among cell types. These proteins seem to be particularly important in developmental processes, such as neuronal migration (Cina et al. 2009; Elias et al. 2007; Fushiki et al. 2003; Wiencken-Barger et al. 2007). In addition, NCC express high levels of connexin43 (Cx43, GJA1) and display it on the cell surface (Boot et al. 2006; Huang et al. 1998; Li et al. 2007; Ruanvoravat and Lo 1992). Knock-down or knock-out of Cx43 reduces NCC migration (Huang et al. 1998; Xu et al. 2006). The deficiency of Cx43 can lead to conotruncal heart malformations and delayed development of craniofacial osteoblasts. In both cases, NCC dysfunction plays a role, as the underlying developmental processes heavily rely on NCC (Huang et al. 1998; Lecanda et al. 2000). Knowing this, it is surprising that most studies so far have not found a connection between NCC migration and GJ functioning. It has been argued that...
GJ-independent functions of connexins are essential for NCC (Francis et al. 2011; Xu et al. 2001, 2006).

Although the connection of PCBs and GJ is well established, and the role of GJ in migration is well-known, the effects of PCBs on normal cell migration (different from tumor invasion) have not been considered. In the present study, we investigate the mechanism of PCB-induced migration-inhibition of NCC, as an example of a non-transformed cell type. We applied here considerations of the adverse outcome pathway (AOP) concept (Leist et al. 2017) to identify a molecular initiation event (MIE) or a key event (KE) of toxicity. We asked whether the approach of multiple structure-activity-relationship (SAR) comparisons would be a new general approach in modern toxicology to identify MIE/KE of AOP. As PCBs have many cellular targets, it is difficult to find the MIE that leads to migration-inhibition. To help finding the mechanism, we made use of SAR considerations. For this purpose, a set of chemically variable PCBs were tested for their effects on NCC migration. We then overlayed the resulting SAR to existing SARs to reduce the number of possible targets. We identified GJIC as a potential cellular target, and we subsequently showed that PCBs disturbing NCC migration also interfere with Cx43 localization.

Materials and methods

NCC differentiation

NCC were differentiated from H9 (WA09; WiCell, Wisconsin) human embryonic stem cells (hESC) modified to express GFP under the control of the Dll1 promoter (H9-Dll1; provided by M. Tomishima, Memorial Sloan Kettering Cancer Centre, New York, NY, USA) as described earlier (Zimmer et al. 2012). Import of the hESC and all experiments utilizing them were carried out according to German legislation and under the license of the Robert-Koch Institute (license number 1710-79-1-4-27).

Differentiation to NCC was performed exactly as described earlier (Nyffeler et al. 2017b; Zimmer et al. 2012). Briefly, the protocol involves differentiation of hESC to rosettes, manual picking of rosettes and FACsorting for positive expression of the NCC marker HNK-1 and negative expression of Dll1. The sorted cells were expanded in N2-S medium, consisting of DMEM/F12 (Gibco) and supplemented with 100 μg/ml apotransferrin, 25 μg/ml insulin, 8.6 mM glucose, 100 μM putrescine, 30 nM selenite, 20 nM progesterone (all purchased from Sigma-Aldrich), 1x GlutaMax and 1% penicillin/streptomycin (both from Gibco), 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml fibroblast growth factor (FGF) 2 (both from R&D). After 27–30 days, cells were cryopreserved until further use. For all experiments, freshly thawed NCC were used and cultured on poly-L-ornithine/fibronectin/laminin coated plates in N2-S medium supplemented with 20 ng/ml EGF and 20 ng/ml FGF2.

NCC migration assay (‘cMINC’)

The NCC migration assay was performed exactly as described in Nyffeler et al. (2017b). Briefly, cells were seeded (95,000 cells/cm²) in poly-L-ornithine/fibronectin/laminin coated 96-well plates containing silicon stoppers (Platypus Technologies, Madison, WI, US) to create a circular cell-free area. One day after seeding, stoppers were removed to initiate migration and medium was refreshed. After 24 h, test compounds were added (in general as 5x concentrate relative to the final test concentration), and after additional 24 h of incubation, migration and viability was measured. For each biological replicate, four technical replicates were performed. For testing of protecting inhibitors, inhibitors were added as 10x concentrate relative to the final test concentration for 1 h, before PCBs were added as 10x concentrate for 24 h. A list of the tested PCBs and inhibitors is given in the Supporting Information.

Image acquisition was performed exactly as described in Nyffeler et al. (2017b) by staining with H-33342 and calcein-AM and imaging with a high-content imaging microscope (Cellomics ArrayScanVTI, Thermo Fischer). Viability was defined as the number of H-33342 and calcein double-positive cells, as determined by an automated algorithm described earlier (Krug et al. 2013; Stiegler et al. 2011). For quantification of migration, a software tool (freely accessible at http://invitrotox.uni-konstanz.de/) was developed to estimate the most likely position of the previously cell-free area (covered by the silicon stopper), to set thresholds for color intensity for both dyes, and to count the number of H-33342 and calcein double-positive cells in the region of interest. If not mentioned otherwise, viability and migration were normalized to untreated or solvent control (0.1% DMSO). In a typical experiment, 2000–2500 viable cells were counted for the control and 150–300 migrated cells were in the region of interest. Concentration-response curves from averaged data (n = 3 experiments) were fitted using R (R Core Team 2015) and the package ‘drc’ (Ritz and Streibig 2005) to a log-logistic function with four parameters as described in Nyffeler et al. (2017a). Effective concentration (EC) values of the fitted curves were estimated (EC90 of viability and EC50 of migration) and the amount of migration at the EC90 of viability was estimated. If the EC90 of viability was above the highest tested concentration, the extent of migration at the highest tested concentration was used instead.
Time-lapse experiment and cell tracking

Cells were seeded under standard cMINC conditions and imaged during the 24-h treatment period as described by Nyffeler et al. (2017a). Live cell imaging was performed using an Axio Observer.Z1 microscope (Zeiss, Oberkochen, Germany) equipped with an AxioCam MRm camera and an incubation system (37 °C, 5% CO₂). Phase contrast images of the migration zone were acquired every 10 min using a 5x objective. After the time-lapse, migration and viability were determined as described in the ‘NCC migration assay’ section to ensure that the toxicants worked as under standard cMINC conditions with exposure in a standard incubator. Cells were manually tracked using the ImageJ plugin ‘Manual Tracking’ in reverse time. Cells were selected for tracking if they were: (1) viable (i.e., not rounded up) at the end of the time-lapse, (2) located in the circular migration zone at the end of the time-lapse and (3) trackable for the entire time-lapse (i.e., did not leave the imaging frame). For each biological replicate, ten individual cells were tracked per condition (and 20–30 cells for the untreated control). The cumulative distance of each cell was normalized to the median of the untreated control population. Statistical significance of differences between treated and non-treated conditions was calculated on the combined data of two biological replicates using a two-sided Wilcoxon rank sum test.

Transwell migration assay

The transwell inserts (pore size 8 μm, polycarbonate membrane, Corning, catalog no. 3422) were pre-coated with poly-l-ornithine/fibronectin/laminin. Cells (100 μl) were seeded at a density of 50,000 cells per insert (150,000 cells/cm²) into the upper chamber. After 1 h, test compounds were added as 5x concentrate (25 μl) to the upper chamber and 600 μl as 1x concentrate to the lower chamber. After 3 h, cells were stimulated to migrate through the membrane by addition of fetal bovine serum (FBS) to the lower chamber (final conc. 5%). The cells were incubated for 6 h at 37 °C and 5% CO₂ and allowed to migrate. After incubation, the medium was aspirated from inserts and reservoirs and then the upper side of each insert was gently swabbed, using cotton swabs, to remove cells that had not migrated through the membrane. Reservoirs and inserts were washed once with phosphate buffered saline (PBS) and then the migrated cells were fixed with 3.7% formaldehyde and stained with crystal violet for 10 min. Then, the inserts were thoroughly rinsed with water and dried for at least 24 h. To evaluate the number of migrated cells through the membrane, five pictures per condition were taken under the light microscope. In a typical experiment, stimulation with FBS led to ca. 100 migrated cells per picture. The number of migrated cells for all data displays was normalized to that of cells stimulated only with FBS in the absence of toxicants. Data are from ≥3 independent experiments. In each experiment, there were two technical replicates (transwell inserts) for the control and one for treatment condition. Data for each technical replicate are based on five imaging fields.

Assessment of migration in various cell types

The human breast adenocarcinoma cell line MDA-MB-231 and the murine fibroblast cell line NIH-3T3 were maintained in DMEM with 4.5 g/l glucose and Glutamax (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. For assessment of migration, cells were seeded (63,000 cells/cm²) into 96-well plates containing silicon stoppers. One day after seeding, stoppers were removed to initiate migration, and medium was refreshed. Toxicants were added as 5x concentrate (in 20% of the final culture volume), and cells were allowed to migrate for 24 h before migration and viability were assessed as described in ‘NCC migration assay’. Cytochalasin D (CytoD), an actin polymerization inhibitor, was used as a positive control to ensure that the conditions allowed to measure inhibition of migration. Two biological experiments were performed with four technical replicates each.

For assessment of PCB cytotoxicity on peripheral blood-derived lymphocytes (PBL), blood donation for research purposes was approved by the local institutional review board, and individual donors gave written consent. Peripheral blood mononuclear cells from healthy donors were enriched by density gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences). Monocytes were depleted from the cell pool with anti-CD14-conjugated microbeads (Miltenyi). The remaining PBLs were cultured in RPMI-1640 medium supplemented with 2% human AB serum, 1% non-essential amino-acids and 0.5% β-mercaptoethanol (Lanza). They were treated with PCBs or vehicle control (DMSO) for 16–20 h. Cells were harvested and stained with TO-PRO3®-3 (ThermoFischer Scientific) to distinguish live from dead cells and quality controlled on an LSRII flow cytometer (BD Biosciences). Chemotaxis of PBL treated with PCBs or vehicle control (DMSO) for 16–20 h was quantified by measurement of migration through a polycarbonate filter of 5 μm pore size in 24-well Transwell® chambers (Corning Costar) as previously described (Hauser et al. 2016). Cell culture medium (600 μl) containing 500 ng/ml of the chemokine CCL21, or medium alone as a control for spontaneous migration, was added to the lower chamber; 10⁶ cells in 100 μl were added to the upper chamber. After 1-h incubation at 37 °C, a 500 μl aliquot of the cells that migrated to the bottom chamber was counted by flow cytometry by acquiring events for a fixed time of 60 s using the Diva software. Specific cell migration was calculated by subtracting the number of cells migrating in the absence of
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chemokines. The number of specifically migrated cells was expressed as percent of input cells and compared to the vehicle control. Viability data are from one biological experiment, whereas chemotaxis experiments were performed with PBLs from three different blood donors.

Quantification of PCB distribution in the well

Three types of samples were produced: (I) PCB (5 µM) in cell culture medium in low-absorbance vessels; (II) solution I in a standard coated cell culture dish; (III) solution I in a coated cell culture dish containing cells. PCB content was measured from conditions II and III after 3 or 24 h. For condition III, cells were cultured exactly as described under ‘NCC migration assay’ with the exception that no silicon stops were introduced. Cells were treated 2 days after seeding with solution I.

PCBs were extracted from the samples by extraction with methyl-, tert-butyl-ether (MTBE). From each sample, 40 µl were transferred into a fresh vial and an equal volume of water containing 0.1% formic acid (Fisher Scientific) was added. The sample was transferred to a supported liquid extraction plate. A positive pressure was applied to load the sample into the plate. MTBE (Fisher Scientific) was added to each well and left to pass through the plate and into a glass insert. The collected sample was dried under nitrogen at 30 °C. Each sample was reconstituted in a volume of MTBE and capped for injection into the gas chromatography–mass spectrometer along with an internal standard (phenanthrene, Sigma-Aldrich). Further analytical details are given in the Supporting Information. To quantify the PCB amount, a serial dilution of solution I was quantified to obtain a calibration curve. PCB content of wells with and without cells was normalized to this calibration sample (considered as 100%). The PCB amount bound to plastic was calculated from the difference I-II. Intracellular PCB amounts were calculated from the difference I-II-III. The experiment was performed three times.

CALUX bioassays for nuclear receptors and stress pathways

The panel of tested cell lines contained the DR CALUX (Garrison et al. 1996), which is a rat H4IIE liver cell based cell line expressing the dioxin receptor (AhR) endogenously. The other selected cell lines are the ERα CALUX and AR CALUX (Sonneveld et al. 2005), and the PR CALUX and PXR CALUX (van der Burg et al. 2015, van der Burg in preparation), all of which are human U2-OS cell lines stably transfected with an expression construct for the human estrogen (ER), androgen (AR), progesterone (PR) or pregnant X (PXR) receptor, and a reporter construct consisting of multimerized responsive elements for the cognate receptor coupled to a minimal promoter element (TATA) and a luciferase gene. The panel was completed with two U2-OS-based singly transfected lines expressing a reporter gene only, designed to selectively measure the activity of the endoplasmic reticulum stress pathway (ESRE CALUX) and the activator protein 1 pathway (AP1 CALUX), respectively (van der Burg et al. 2013). The Cytotox CALUX, used as a control line for non-specific effects, consisted of human U2-OS cells stably transfected with an expression construct constitutively expressing the luciferase gene, and is described in van der Linden et al. (2014).

Cells were maintained as described previously (Sonneveld et al. 2005). The automated CALUX assays were carried out as described earlier (van der Burg et al. 2015). In brief, the assay was performed in assay medium, consisting of DMEM without phenol red indicator (Gibco) supplemented with 5% DCC-stripped FBS, 1× non-essential amino acids (Gibco) and 10 µg/ml streptomycin. A cell suspension in assay medium was made of 1×10⁵ cells/ml, and white 384-wells plates were seeded with 30 µl cell suspension/well. After 24 h, exposure medium was prepared. A dilution series in 0.5log unit increments of each test compound (in DMSO) was added to a 96-well plate containing assay medium. Of this exposure mixture, 30 µl was added to the assay plates containing the CALUX cells. Additionally, DMSO blanks and a full concentration response curve of reference compounds were included on each plate. The final solvent concentration in the well was 1%. All samples were tested in triplicates. The preparation of the compound dilution series as well as the exposure of the cells were performed on a Hamilton Starlet liquid handling robot coupled to a Cytomat incubator. To be able to detect receptor antagonism, the AR- and PR CALUX assays were performed in antagonistic mode. The assay procedure was as described above, with the only exception that the EC50 concentration of the reference agonist was present during the exposure.

After 24 h exposure the exposure medium was removed using an EL406 washer-dispenser (BioTek) and 10 µl/well triton lysis buffer was added by the EL406. Subsequently, the luciferase signal was measured in a luminometer (InfinitePro coupled to a Connect Stacker, both TECAN).

The luminometer data was analyzed as following: the average of the triplicate wells was determined, and the average blank (DMSO) value was subtracted. The maximum response elicited by the reference compound was set to 100% (full receptor- or pathway activation), and the other values were scaled accordingly. Graph Pad Prism was used to fit a sigmoidal curve through the data (four parameters, variable slope). The EC10 concentration was defined as the concentration where the response elicited by the test compound equals 10% of the maximum response of the reference compound. For antagonist experiments, EC20 values were
determined instead, which were defined as the concentration where the test compound causes 20% inhibition of the basal signal elicited by the receptor agonist which was set to 100%.

Literature research

The literature was searched for structure-activity-relationship studies of PCBs with various cellular targets and key events (KE). Results of PCBs with 4 to 8 chlorine substitutions (e.g., PCB40–PCB205) were considered. Tested PCBs were categorized as active/inactive based on the conclusions in the respective publications. For two publications, we developed our own categorization scheme: for CYP1A1 induction and PXR activation in Al-Salman and Plant (2012), PCBs were considered active if they led to > twofold induction. For PXR activation in Tabb et al. (2004), PCBs were considered active if the readout was significantly increased (at the 5% level) or if the fold change was > 5. For PXR antagonism in the same study, PCBs were considered active if Ki was < 9 µM.

Parachute assay to measure gap junctional communication

Cells were seeded (95,000 cells/cm²) in PLO/fibronectin/laminin coated 24-well plates and cultured under standard cMINC conditions with 600 µl medium/well. After 2 days in culture, treatment was initiated. Four hours before quantification of the assay endpoint, donor cells were labeled. For this, cells kept in parallel under control conditions were stained for 30 min with calcein-AM (533 nM final conc.; Sigma). After two washing steps with pre-warmed PBS, 150 µl HyClone HyQTase (GE Healthcare Life Sciences) was added for 20–30 min at 37 °C to detach the cells. Then, cells were washed [medium (450 µl)] was added, cells were centrifuged and supernatants were removed, followed by resuspension in 600 µl of fresh medium. An aliquot (6 µl + 54 µl fresh medium) of this donor population was added to each well with acceptor cells (i.e., various treatment conditions). Under these conditions, the donor:acceptor ratio was about 1:100. Dye-transfer was allowed to occur for a total of 3 h. During the last 30 min, 1 µg/ml H-33342 was added, to label cell nuclei. Imaging was performed on a high-content imaging microscope (Cellomics ArrayScan-VTI, Thermo Fischer) equipped with a Hamamatsu ORCA-ER camera of 1024 x 1024 pixel resolution run at 2 x 2 binning mode. Of each well, 49 fields were imaged using a 5x lens.

The obtained images were exported to TIF format and analyzed using R (R Core Team 2015) and the package ‘EBImage’ (Pau et al. 2010). In a first step, the calcein image was normalized, background and gamma corrected. Donor and acceptor cells were defined based on pixel brightness. A watershed algorithm defined the regions with (parachuted) donor cells and their acceptor area as objects. Objects with donor cells < 60 or > 500 px or with an acceptor area > 3000 px or too low nuclei density (based on the H-33342 channel) were excluded. From the remaining valid objects, the ratio of the area (in px) of acceptor cells and the corresponding donor cell was calculated and compared between treatments. To this end, the values of all untreated controls (usually three wells with 49 pictures each) were pooled and the median calculated. For each treatment well, the percentage of ratios greater than the median of the untreated control was calculated and normalized to the untreated control. Data displayed are from three biological experiments with > 100 valid objects per experiment.

Immunofluorescence staining

For immunocytochemical staining, cells were fixed for 20 min by adding one volume of 4% formaldehyde and 2% sucrose in phosphate buffered saline (PBS) to the medium. For intracellular epitopes, cells were permeabilized for 15 min with 0.1% Triton, followed by blocking with 10% FBS in PBS for 1 h before primary antibodies were applied in 10% FBS in PBS overnight. The next day, cells were washed with 0.05% Tween in PBS and secondary antibodies were applied for 1 h. Cell nuclei were counterstained with 1 µg/ml H-33342 for 10 min. Images were acquired using a laser scanning confocal microscope (Zeiss LSM 700, Zeiss, Oberkochen, Germany) or an Axio Observer.Z1 microscope (Zeiss, Oberkochen, Germany). The list of antibodies and their dilutions can be found in the Supporting Information.

Quantification of connexin43 plaques (Cx43pq)

Cells were seeded (95,000 cells/cm², 315 µl medium/well) in PLO/fibronectin/laminin coated 8-well µ-slides with glass bottom (iBidi, Munich, Germany). After 1 day, medium was refreshed (320 µl). After another day, test compounds were added (80 µl, 5x concentrate; for 0.5–3 h) before cells were fixed and stained for Cx43 as described in ‘Immunofluorescence staining’. Images were acquired manually on an Axio Observer.Z1 microscope (Zeiss, Oberkochen, Germany) equipped with an Axiocam MRm camera and appropriate filters using the 20x lens by focusing on the connexin43 plaques (Cx43pq) and imaging H-33342 with an offset of ~ 8 µm. Exposure time for connexin43 was chosen in a way that the plaques were overexposed, but not other cellular structures. Images were exported to TIF format and analyzed using R (R Core Team 2015) and the package ‘EBImage’ (Pau et al. 2010). Images were thresholded to display only pixels with an intensity > 0.8 (on a normalized scale of 0–1), followed by segmentation of the remaining objects. Objects
with an area \(<\) 10 pixels (px) were counted as connexin43 plaques (Cx43pq). A typical image of a solvent control culture (512×512 px; i.e., about 400 \(\mu\)m wide) contained approximately 50 such objects. For each condition, 10–15 images were analyzed, averaged, and normalized to the solvent control. Data displayed are from at least two independent biological experiments.

Data handling and statistics

If not mentioned otherwise, values are expressed as means ± SD from three independent experiments. Independent experiments are here also called 'biological replicates' (Aschner et al. 2017; Leist et al. 2010) and they are defined as using different cell preparations (while technical replicates here correspond to different cell culture wells within one experiment). The statistical methods (parametric and non-parametric) were adapted to the questions behind the experiment and are specified in the figure legends. Calculations were done in Excel and R, for data display GraphPad Prism and R were used.

Results

Differential effects of PCBs on NCC migration

Previous studies had shown that some diortho chlorinated PCBs can inhibit NCC migration (Dreser et al. 2015; Nyffeler et al. 2017b). Here we asked whether this was a general feature of PCBs. For this purpose, we chose a diortho PCB (PCB180), a monoortho (PCB118) and a nonortho (PCB77) chlorinated congener (Fig. 1a), and they were tested in three different migration assays.

The first test was a transwell migration assay. Under control condition, NCC migrates towards a chemoattractant stimulus (serum) within 6 h. Upon pre-treatment with PCB118 and PCB180, transwell migration was drastically reduced in a concentration-dependent manner, while PCB77 did not inhibit transwell migration in a similar concentration range (Fig. 1a). These data suggest that PCBs have differential effects on NCC migration. To confirm the findings by direct observation, the migration speed of single cells was determined by a method as described earlier (Nyffeler et al. 2017a, b). For this purpose, cells were seeded in 96-well plates containing silicon stoppers that create a circular cell-free area. Upon removal of the stoppers, NCC started to migrate into the cell-free area (Fig. 1b). PCB118 and PCB180 decreased the traveled distance of single cells (e.g., the average cell speed in 24 h) by approx. 50% (Fig. 1c). These experiments also showed that the PCB exposure slowed down the cells already after few hours (data not shown), while the nonortho chlorinated PCB77 did not affect cell speed at any time. Analysis of NCC migration using high-content imaging, and quantification of the number of migrated cells (Nyffeler et al. 2017a, b) confirmed these findings. While PCB77 did not affect migration at concentrations up to 40 \(\mu\)M, PCB118 and PCB180 inhibited migration at low micromolar concentrations and in a concentration-dependent manner that did not affect the number of viable cells (Fig. 1d). Altogether, these results indicated that not all PCB congeners interfere with NCC migration.

Negligible effects of PCBs on the migration of other cell types

We wondered why the strong effects of PCBs on cell migration had received little attention earlier. Therefore, we investigated whether other migratory cell types would also be affected in a similar way as NCC. For this purpose, a human breast cancer cell line (MDA-MB-231), and mouse embryonic fibroblasts (NIH-3T3) were chosen as well-established models for cell migration. A modified version of the CMINC high-content imaging setup was used to quantify cell migration over a 24-h period. Under these conditions, the positive control cytochalasin D strongly inhibited migration, while PCB118 and PCB180 neither affected migration nor viability (although concentrations up to 25 \(\mu\)M were tested, Fig. 2a). To follow-up on these negative findings, we chose peripheral blood lymphocytes (PBL) as a primary cell type known to be very motile. The latter cells typically migrate at much higher speed than NCC, and they were therefore tested in a 1 h transwell setup (using the well-established chemokine CCL21; Hauser and Legler 2016). Cells exposed to PCB180 (25 \(\mu\)M) migrated to the same extent as under control conditions, even if they were pre-treated with the toxicant for 20 h (Fig. 2b).

These results suggest that the migration-inhibitory effect of PCBs is limited to a few cell types. Concerning the effects observed here for NCC, the cell type specificity may be due to the interference of PCBs with a cellular process particularly important for this fetal cell population, but not for general cell migration. Alternatively, PCBs might act by binding to a ‘receptor’ expressed by NCC, but not by other cell types.

Bioavailability of migration-inhibiting PCBs

PCBs have been used for mechanistic studies in a broad concentration range. An in vivo single digit micromolarity seems to be feasible (Choi et al. 2003; Jensen 1989; Wässermann et al. 1979), and is considered realistic in recent studies (Pierucci et al. 2017; Ziegler et al. 2017), while concentrations clearly beyond this limit pose experimental problems (solubility) and may not be toxicologically relevant. We observed here that PCB118 and PCB180 strongly inhibited migration at 5 \(\mu\)M. We were interested in whether
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this nominal concentration (Groothuis et al. 2015) roughly corresponded to the total concentration of PCB in cell culture. Therefore, the PCB contents in the different compartments of a culture dish (in the cell supernatant, bound to plastic and intracellular) were assessed by chemical analysis. To this end, cells were incubated with 5 µM PCB for 3 or 24 h. It became apparent, that after 3 h, 25–50% of PCB bound to the cell culture dish plastic (Fig. 3). This fraction increases to around 75% after 24 h. This means that the available PCB concentration was 25–75% of the nominal concentration, i.e., about 1.2–3.8 µM, when 5 µM were tested. Additionally to PCB118 and PCB180, several non-ortho, mono-ortho, di-ortho and tri-ortho PCBs were tested for their binding to cell culture dishes. After 3-h incubation, about 60% of PCB (PCB 126, 169, 95, 132, 110, 153, 167) bound to the cell culture dish plastic. The fraction bound to plastic was similar for all tested PCBs, and therefore the real available PCB concentration in the cell culture medium was about 2 µM instead of the nominal concentration of 5 µM. Some PCB may have accumulated also in the cells. However, this was less than 10% of the total amount (and associated with a large variance). It could therefore not be exactly quantified. In conclusion, the cells were exposed to extracellular PCB concentrations lower than the nominal concentrations, but still within the same order of magnitude. Since plastic adsorption played an important role, these data suggest that experimental standardization (e.g., consistent conditions, culture ware, etc.) was highly important for our study.

Investigation of important cell functions as potential targets of PCBs

One explanation for apparently specific inhibition of NCC by PCBs could be that these cells are particularly sensitive to PCB toxicity. Although we found that general viability measures were not affected at concentrations that inhibited migration, some more subtle effects may have remained undetected. A cell function that is possibly more important for migration (in the time frame of the cMINC assay) than for overall viability is the correct and efficient production and intracellular transport of proteins (especially to and from the cell membrane). Glycosylation of proteins is a critical function of the biosynthetic-secretory pathway that may be used as functional readout (Arvan et al. 2002; Ferris et al. 2014). Therefore, we investigated whether PCBs affected this cell biological process. For the measurement, we used a well-established method from the field of metabolic glycoengineering (Prescher and Bertozzi 2005): we fed cells with a modified sugar precursor, and subsequently quantified how efficient this chemical biological tool was incorporated as sialic acid into the pool of membrane glycoproteins (Du et al. 2009; Kang et al. 2015; Niederwieser et al. 2013). The assay had a large dynamic range for NCC, and use of positive controls (tunicamycin and berbelfidin A, known inhibitors of the N-glycosylation pathway) showed a very pronounced inhibition of the assay endpoint, independent of overt cytotoxicity (Fig. S1). None of the tested PCBs (PCB118 and PCB180) affected the display of the metabolized and incorporated sugar (sialic acid) on the plasma membrane (Fig. S1). Thus, many complex cellular processes, requiring Golgi functioning, protein trafficking and glycoprotein synthesis, seemed to be unaffected by exposure to PCB.

As an additional approach, the cellular status was examined by microscopy and by quantification of gene
expression. Overall cell morphology and cytoskeletal structure remained within the range of normal variations, and the expression of several differentiation markers (e.g., nestin) remained unchanged upon treatment with PCB180 (10 µM) (Fig. S2A). Moreover, cadherins and integrins (cell–cell and cell–matrix receptors involved in cell migration), were neither up- nor down-regulated upon PCB180 treatment (Fig. S2B, C).

Next, we investigated whether targets of PCBs known from the literature would qualify as candidates for MIE, i.e., whether they were responsible for the adverse outcome in our test (functional impairment of NCC with regards to migration; Fig. 4a). As PCBs are known to directly interact with the AhR, the RyR, and several nuclear receptors, we tested whether agonists and antagonists of these receptors affected NCC migration. Ligands of the AhR did not show effects in the cMINC assay (Fig. 4b), even though the receptor appeared to be biologically active (target genes were induced by the dioxin-like non-ortho-chlorinated PCBs 77, 126 and 169; Fig. S3A). RyR also had no effect on migration, and the same was true for thyroid receptor (THR) ligands (Fig. 4b). In a further series of experiments, we investigated whether receptor modulation affected the migration-inhibitory effect of PCB180. Neither AhR, nor RyR or THR modulation blocked the effect of the ortho-chlorinated PCBs (Fig. 4c). Additionally, several approaches to antagonize retinoic acid receptors failed to show an effect (Fig. 4b, c). To gain evidence on whether PCBs may act as agonists on retinoic receptors, we compared the regulation of the receptors [which are downstream genes regulated by the retinoic receptor protein complexes (Bagamasbad and Denver 2011)] to the ones triggered by retinoic acid (RA) itself. While RA showed the expected responses, PCB180 had no effect (Fig. S3B).

Taken together, these data suggest that neither the AhR nor RyR, THR, and retinoic acid receptors are involved in PCB-induced migration-inhibition of NCC. We therefore developed an alternative strategy to identify potential targets that were affected by PCBs, and that mediated their effect on NCC migration. We reasoned that correlation of effects of a couple of compounds would not be sufficiently defining, given the multiple and complex cellular reactions towards PCBs (problem of overfitting). Therefore, we decided to test a larger number of PCBs, and to determine the SAR of PCBs concerning cMINC outcomes. We planned to compare this SAR with various PCB SARs known from the literature. Comparison of these SAR was expected to help to identify the most likely candidates for cellular processes affected by PCBs and being involved in migration-inhibition (Fig. 4d).
4.3 Results

These data are in line with those obtained earlier (Fig. 1), but the few tested concentrations (lower throughput than the cMINC) did not reveal striking differences in efficacy. The most parsimonious and robust SAR-model supported by both assays is that “at least one ortho-chlorine is required for PCBs to inhibit NCC migration”.

**Comparison of PCB effects on NCC migration with other PCB SARs**

Having established a SAR for migration, we followed the strategy outlined in Fig. 4d and searched for a feature that has the same SAR. For this purpose, a binary classification (active/inactive) was used to calculate the correlation of various features with cMINC data (Fig. 6a). First, we checked, whether simple physico-chemical descriptors (apart from biphenyl bond rotation angle and related parameters) indicated active or inactive PCB. However, molecular weight (MW), hydrophobicity (logP), apolar desolvation and polar desolvation energy did not differ significantly between the active and inactive PCBs (Fig. S6).

Next, we searched in the literature for data sets describing the effects of planar (non-ortho chlorine) vs non-planar (ortho-chlorine) PCBs on different receptors and cellular processes to calculate the correlation with cMINC data (Fig. S7). To complement the legacy data that showed sometimes large heterogeneity between assays and studies, we selected a subset of compounds for direct comparative measurements (Fig. S8). For generation of these data, reporter assays on the basis of luciferase expression in CALUX cells were used (Garrison et al. 2015, 2013; van der Burg et al. 2015, 2013; van der Linden et al. 2014). All reporter constructs were integrated in the same cell line to allow comparison not only within assays, but also across different endpoints (Fig. 6b). Testing of PCBs in the CALUX system confirmed that concentrations up to 10 µM were relatively well tolerated by cells. Moreover, measurements of two stress responses (endoplasmic reticulum stress and AP-1 activation) (van der Burg et al. 2013) showed that PCBs trigger such responses only at cytotoxic concentrations, but not in the non-cytotoxic range (Fig. S8). Thus, receptor activation information in our data set is most likely specific and independent of general cell toxicity.

The SAR on AhR activation and CYP1A induction reported in the literature (Al-Salman and Plant 2012; Hestermann et al. 2000) clearly did not match the SAR on NCC migration. Planar, non-ortho chlorinated PCBs are known to activate the AhR (Safe 1994), and these congeners were inactive on NCC migration. Our own data confirm the large activity difference (1000-fold) between highly potent (sub-nanomolar) planar and less potent non-planar PCB on CALUX cells (Fig. S8). Notably, these measurements also show that some (but not all) ortho-chlorine PCBs may

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**Fig. 3** Distribution of PCB in the culture dish. A PCB-solution (5 µM) in cell culture medium was prepared in a low-absorbance vial, and its actual PCB content quantified and taken as 100% reference value. Then, this solution was added to cell culture wells that were otherwise empty or that contained NCC under standard assay conditions. After 3 or 24 h, samples were taken from the medium and assayed for PCB content. From the values obtained, the fractions of PCB absorbing to plastic (corresponding to the loss in dishes not containing cells), or accumulating in cells (difference of theoretical PCB content and the sum of content in supernatant and on plastic) was calculated. An example for reading the data is given for the 3 h incubation with PCB118: of the 5 µM added, about 47% were recovered from the cell supernatant (remaining concentration of 2.3 µM), 46% of the toxicant adsorbed to plastic and about 7% was accumulated in cells. The experiment was performed three times and means ± SD are shown.
activate the AhR in the submicromolar concentration range. Concerning the NCC, we found that migration-inhibiting non-planar PCBs did not increase CYP1 (downstream response of AhR activation) expression in NCC (in contrast to planar PCBs) (Fig. S3A).

The reported SAR for RyR binding and activation (Fritsch and Pessah 2013; Pessah et al. 2006) showed a good correlation with the NCC migration SAR. But as RyR agonists and antagonists did not affect NCC migration (Fig. 4b, c), RyR is unlikely to be the MIE for migration-inhibition in NCC.

Concerning nuclear receptors for steroids, several PCB SAR studies have been performed. Data are available for the AR, ER, THR, PXR and constitutive androstane receptor (CAR) (Al-Salman and Plant 2012; Hamers et al. 2011; Tabb et al. 2004). Literature and our own data on AR antagonism correlate well with cMINC data, while ER and related targets showed a poor correlation. AR antagonism was considered to be unlikely as MIE here, as stimulating ligands of the AR were not present in the test system. The same applies to PR antagonism. Concerning THR and retinoic receptors,
Fig. 5 Structure–activity relationship of PCBs and NCC migration. 

(a) Concentration–response curves of selected PCBs: the cMINC assay was used as in Fig. 1 to determine viability (open triangles) and migration (colored circles) under different experimental conditions. All data are normalized to those of untreated controls. For better visual comparison, the horizontal gray dotted line was plotted at 100%; the vertical gray dotted line is at 2 µM. Data are expressed as means ± SD from three independent experiments. Chemical structures are indicated with chlorine residues marked in purple (ortho position) or green (meta and para positions).

(b) Overview of the effects of all tested PCBs on viability and migration. A log-logistic function with constraints was fitted to the concentration–response data of 26 PCBs. From these curves, the EC50 of migration and the % migration at the EC90 of viability were determined and plotted against one another. For some PCBs, the EC90 of viability was not reached; in these cases, the migration at the highest tested concentration is shown. The color-shaded fields indicate areas of the scatterplot that are likely to harbor non-ortho PCB, mono-ortho PCB, di-ortho PCB, etc.

(c) Effects of selected PCBs on individual cell speed. Time-lapse video microscopy was performed during the toxicant exposure, and the migration track of individual cells was identified. For each treatment condition, 8–12 individual cells were tracked and each cumulated travelled distance was normalized to the solvent control. Data from two biological replicates were pooled. The box represents the first and third quartiles, the black line the median (number given in the rectangle). The color code indicates the number of ortho-chlorine substituents. Statistical significance of differences between treated and non-treated conditions was calculated using a Wilcoxon rank sum test. Statistical indicators are given below the boxes: *** p < 0.001. (Color figure online)
Fig. 6 Correlation of biochemical features of PCB with NCC toxicity. a Schematic representation of feature comparison. In a first step, a large group of PCBs is characterized as being active or inactive concerning effects on NCC migration. In a second step, literature data are obtained for the same PCB concerning various features that relate to a key event of a toxicity pathway/adverse outcome pathway (e.g., activation of the estrogen receptor). In the third step, the correlation of NCC activity and key events is examined for several candidate features (here shown as X, Y, Z, with correlations for the examples being indicated below). b A binary classification scheme was chosen to examine feature correlation. For each feature (with literature references indicated), it was determined, how many (#) ortho PCB or non-ortho PCB were active or inactive. The correlation score (here called ‘correct SAR’) was calculated as: 100 × (#inactive non-ortho + #active ortho)/total # of PCB tested. The extent of correlation was color-coded (red: very good; blue: very poor). Abbreviations/feature definitions: AhR, aryl hydrocarbon receptor; Cyp1A1, cytochrome P4501A1; EROD activity, inhibition of cytochrome P450 (mainly Cyp1A family) enzymatic activity; RyR, ryanodine receptor; AR, androgen receptor, ER, estrogen receptor; E2SULT, estradiol sulfotransferase, TTR, transthyretin, PAR, pregnane X receptor, CAR, constitutive androstane receptor, DAT, dopamine transporter, GJ, gap junctions. (1) Hestermann et al. (2000); (2) Al-Salman and Plant (2012); (3) Pessah et al. (2006); (4) Fritsch and Pessah (2013); (5) Hamers et al. (2011); (6) Tabb et al. (2004); (7) Shain et al. (1991); (8) Mariussen et al. (2001); (9) Wigestrand et al. (2013); (10) Hemming et al. (1991). (Color figure online)
the SAR correlated poorly, and our functional data also did not support a role of these receptors (Fig. S3, Fig. 6). The same applied to the vitamin D receptor (VDR), for which we measured the induction. Several PCBs increased VDR expression levels, but since two non-ortho PCBs were active, while the three mono-ortho PCBs were inactive, we concluded that the VDR is not a likely MIE in NCC (Fig. S3C).

The SARs for CAR and PXR activation were very similar as the one for NCC migration (Fig. 6, Fig. S7, Fig. S8). This may be worth some further exploration in the future, but for the present study, we considered the likelihood of e.g., PXR being the MIE responsible for impaired migration as low. This is based on measurements of the expression level of the receptor on mRNA level: no transcripts were detectable in NCC (data below detection limit; not shown).

Several SARs for interference with the dopamine system have been reported (Mariussen et al. 2001; Shain et al. 1991; Wigestrand et al. 2013), all of them with good overlap to the SAR of NCC migration (>90% overlap). We ranked the likelihood of this mechanism as MIE as low, as NCC neither express tyrosine hydroxylase for synthesis of dopamine, nor do they express dopamine transporters (Zimmer et al. 2012).

Finally, PCBs are known to interfere with gap junctional intercellular communication (GJIC). Two studies reported a SAR, and in both cases, there was a high overlap (>95%, only one mismatch) (Fig. 6). As NCC are known to express high levels of the gap junctional protein Cx43, and as GJIC is a well-established NCC feature (Boot et al. 2006; Huang et al. 1998; Li et al. 2007; Ruangvoravat and Lo 1992), we further investigated this hypothesis (see below).

Our approach of comparing SAR for different processes has several obvious weaknesses. Even though we did not match the numbers of actives and non-actives or weighted the data according to their uncertainty level, this coarse approach yielded some useful information that allowed ranking of likely candidate targets for PCBs, concerning causal involvement in cMINC effects. The GJIC was an initially surprising correlation, but considering all background information, it was regarded possible and sufficiently plausible to be further investigated.

**Investigation of gap junctional intercellular communication as target of PCB**

To measure GJIC in NCC, dye-transfer among neighboring NCC was assessed using the ‘parachute assay’ (adapted from Lecanda et al. 2000). NCC were cultured under standard cMINC conditions and treated with the PCBs for 24 h (Fig. 7a). Three hours before read-out, calcine-loaded (but otherwise untreated) donor NCC were parachuted (dropped as single cells) on top of the treated (acceptor) cultures (Fig. 7b). The assay is based on the fact that calcine spreads from donor to acceptor cells within 3 h, if GJIC is functional.

The extent of dye-transfer into the acceptor layer was quantified using high-content imaging (Fig. 7c). Using this setup, the well-established GJIC blocker carbenoxolone showed the expected inhibitory effect (Fig. 7d). Additionally, PCB180 was found to strongly inhibit dye-transfer of NCC: only about 2% of donor cells spread their dye to PCB-treated acceptors. Altogether 26 PCBs were tested using this assay. Indeed, the non-ortho PCBs did not inhibit GJIC, while 11 of the ortho-chlorinated PCBs were efficient GJ inhibitors. However, 12 of the 23 migration-inhibiting PCBs did not inhibit GJIC (Fig. 7d). Obviously, the SAR concerning GJIC did not correlate well with inhibition of migration, and GJIC was therefore discarded as KE for migration-inhibition by PCBs.

In NCC, block of gap junctions may only occur with highly chlorinated PCBs, as the effect was observed with all compounds that had ≥2 ortho-chlorines plus ≥2 meta-chlorines (with one exception to that rule). The biochemical basis for this effect is unclear at present, but it may be linked to the higher hydrophobicity of these compounds. This may favor other interactions than those to the primary gap junction proteins (connexins). It is firmly established in the field of GJIC that many connexins themselves, but rather affect their localization and post-translational modifications indirectly (Willebrords et al. 2017). For this reason, we still investigated other endpoints (e.g., gap junction protein localization) connected to gap junction structures.

**Changes in Cx43 localization as target of PCB for inhibition of NCC migration**

Cx43, the major connexin expressed in NCC, has been reported to affect NCC migration independent of its role in GJIC (Francis et al. 2011; Xu et al. 2001, 2006). To investigate whether PCBs may affect Cx43, we used immuno-cytochemical methods to investigate whether the toxicant affected protein localization. Confocal imaging revealed that Cx43 is localized mainly to the cell membrane and the Golgi. In untreated NCC, membranal Cx43 was mainly assembled in plaques (Cx43pq), visible as bright spots on confocal images. Upon treatment with PCB180, the Cx43pq dissolved: Cx43 distributed more homogeneously along the cell membrane, and more of the protein seemed to be localized to the Golgi compared to controls (Fig. S9). Moreover, in silico docking and binding affinity prediction methods suggested that PCB180 and PCB153 bind with slightly higher affinity (39–40 kJ/mol) to Cx43 than, e.g., the planar PCBs 77 and 126 (35–36 kJ/mol). For this reason, we felt that this should be explored further, and we established a high-content imaging assay for Cx43pq quantification.

Our approach was based on immunofluorescence staining of NCC layers for Cx43. A thresholding plus segmentation
algorithm was used to identify small, highly fluorescent protein dots. The number of these dots per image field was quantified (Fig. 8a). Using this assay, we found that the gap junction blocker carbenoxolone quickly (within 2–3 h) reduced the Cx43pq, and the same effect was observed with the migration-inhibitory PCBs 180 and 118 within a similar...
Fig. 8 Connexin43 localization and quantification of ‘connexin43 plaques’. a Quantification algorithm of Cx43pq. Cells were stained (H-33342) for DNA (blue nuclei) and connexin43 (red) and imaged on an epifluorescence microscope. The connexin43 channel was thresholded to only display the brightest pixels (> 80% of maximal intensity, displayed here in inverted mode), followed by a segmentation and size (area) filtering of the bright spots (here displayed in black). Objects of < 10 pixels size were scored (= valid objects). For visualization, the valid objects (red) were overlaid on the greyscale connexin43 image. b Example for toxicant-induced changes in the number of Cx43pq (small bright dots). Cells were seeded on glass bottom plates under cMINC assay conditions. They were exposed to test compounds for the times indicated, before they were fixed and stained. Then, the quantification procedure detailed above was applied, and data were normalized to solvent controls. c Quantification of Cx43pq upon treatment with various concentrations of exemplary toxicants. The experiment was performed as in b, but all quantifications were performed after 3 h. d Graphical representation of the experimental setup for the Cx43pq assay: Cells were seeded on glass bottom plates under cMINC assay conditions. They were exposed to test compounds for the times indicated, before they were fixed and stained. Then, the quantification procedure detailed above was applied, and data were normalized to solvent controls. e Quantification of Cx43pq upon treatment with various concentrations of exemplary toxicants. The experiment was performed as in b, but all quantifications were performed after 3 h. f Graphical representation of the experimental setup for the Cx43pq assay: Cells were seeded on glass bottom plates under cMINC assay conditions. 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time scale and to the same extent (Fig. 8b). This fast effect of PCBs is well in line with our observations from cell tracking, that PCB treatment slowed down cell movement within few hours. GJIC-independent effects of Cx43 that could be affected are, e.g., the role of the protein for the stability of leading processes of migrating cells (Elias et al. 2007).

Recording of concentration–response curves revealed, that the reduction of Cx43pq follows the same concentration dependency as inhibition of migration in the cMINC. For instance, the 4-ortho chlorinated PCB104 was more potent than PCBs 180 and 118 (Fig. 8c). Encouraged by these findings, our whole set of PCBs was screened in the Cx43pq assay at a fixed time point (3 h) (Fig. 8d).

All migration-inhibiting PCBs reduced Cx43pq, whereas the three non-ortho PCBs did not have an effect (Fig. 8e). Thus, the observed SAR for Cx43 localization was identical to the SAR for NCC migration-inhibition. This is not necessarily due to direct binding of PCB to Cx43, as in silico estimation of binding energies of all PCBs to Cx43 did not reveal a correlation between predicted affinities and inhibited migration or reduced Cx43pq (not shown). Nevertheless, the correlation of the SAR across compounds between cMINC results and the Cx43pq assay (Fig. 9) was statistically significant (p < 0.0004 for a random association), so that we concluded Cx43 mislocalization represents an early KE involved in the PCB-induced inhibition of NCC migration.

Discussion

We tested PCBs, as a group of environmentally relevant toxicants, and found that all tested ortho-chlorinated PCBs inhibited NCC migration, whereas non-ortho chlorinated, dioxin-like PCBs were ineffective. As investigation of several pathways known to be targeted by PCBs did not reveal the mechanism of migration-inhibition, we employed a new strategy and established a SAR for migration-inhibition and compared it to the literature data. According to the literature, GJIC appeared initially as a process that might explain the effects of PCB on NCC migration, but the SAR for GJIC had only an overlap of 50% with the SAR for migration-inhibition (Fig. 9). However, examinations of GJIC let us consider Cx43 localization. The SAR for this feature was identical to the one for migration-inhibition. Therefore, Cx43 localization seems to be a mechanistic step tightly linked to NCC toxicity of PCBs.

Previous studies found that PCB153 reduces localization features of Cx43 at the plasma membrane of epithelial cells (Kang et al. 1996; Simeckova et al. 2009). In the present study, we found that all tested ortho-chlorinated PCBs reduced Cx43pq in human NCC. Our observations do not provide proof that Cx43 mislocalization is causing the observed inhibition of migration, but the probability that the two SARs would overlap completely by chance is < 0.0004.

To our knowledge, there are no other reports about Cx43 mislocalization in the context of migration-inhibition. However, several studies have reported that knock-down or knock-out of Cx43 reduces migration of NCC (Huang et al. 1998; Xu et al. 2006) and neural cells (Cina et al. 2009; Elias et al. 2007; Fushiki et al. 2003; Wiencken-Barger et al. 2007). Several studies also concluded that Cx43 affects NCC migration in a GJ-independent manner (Francis et al. 2011; Xu et al. 2001, 2006). The C-terminal tail of Cx43 was reported to be important for promoting migration in neural cells (Bates et al. 2007; Behrens et al. 2010; Cina et al. 2009; Crespin et al. 2010; Elias et al. 2007). Francis et al. (2011) showed, that NCC expressing a Cx43 variant lacking the C-terminal tubulin-binding domain (Cx43ΔT) tend to migrate less. Other studies with NCC and other cell types concluded, that Cx43 knock-out cells fail to realign their microtubule organizing center and Golgi in the direction of migration. Additionally, loss of stabilized microtubules and alterations in the actin skeleton were observed (Francis et al. 2011; Rhee et al. 2009; Xu et al. 2006).

The present experiments do not allow conclusions on whether Cx43 is the primary target of PCBs in NCC, and whether the reduction of Cx43pq is causal to the adverse outcome of reduced migration. Alternatively, reductions of Cx43pq may be a biomarker for another, yet unknown KE triggered by PCBs. For example, blocking of cell adhesion

<table>
<thead>
<tr>
<th>Feature</th>
<th>non-ortho</th>
<th>ortho</th>
<th>correct SAR (%)</th>
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<tr>
<td>inhibit migration (cMINC assay)</td>
<td>0</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>decrease cell speed</td>
<td>0</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>inhibit gap junctional communication</td>
<td>0</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>decrease Cx43pq</td>
<td>0</td>
<td>3</td>
<td>25</td>
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Fig. 9 Overview of structure–activity relationships of PCBs. The tested PCB had similar effects on migration and on connexin43 plaques (Cx43pq; 100% correlation). A high correlation with reduced cell speed was also observed (but only about half of the compounds were tested). In contrast to this, inhibition of gap junction communication (parachute assay) did not correlate well with migration or with Cx43pq.
or knock-out of ephrin-B1 can lead to relocation of Cx43 (Davy et al. 2006; Guo et al. 2003). Moreover, Cx43 can bind to the tight junction protein ZO-1, and interaction with ZO-1 can affect GJ plaque size, trafficking and turnover of Cx43 (Giepmans and Moolenaar 1998; Hunter et al. 2005).

At present, it is not entirely clear how PCBs lead to the reduction of Cx43p, and whether it is likely that they bind directly to Cx43. Our in silico docking studies did not find a clear correlation of calculated PCB-Cx43 binding energies and neither migration or plaque formation. Although this approach is associated with several uncertainties, it suggests that it is likely that other or additional targets are affected by PCB in NCC. Such alternative binding partners could lead to a reduction of Cx43p, either by promoting transport away from the membrane or by attenuating the transport to the membrane. In the first scenario, either internalization and degradation via lysosomes, or proteasomal degradation, leading to destabilization of phosphorylated Cx43 at the membrane or endoplasmatic reticulum-assisted degradation might be enhanced (Laird 2005; Qin et al. 2003; Simeckova et al. 2009). In the second scenario, less protein production or less protein transport to the membrane could be the cause. Moreover, Cx43p could be reduced if Cx43 is present at the membrane, but not assembled in plaques.

In our experiments, there was no evidence for disturbed protein production. Firstly, mRNA levels of Cx43 were not affected by PCB treatment (Fig. S10B). Secondly, in immunofluorescent stainings, Cx43 was clearly visible, but seemed to be more accumulated in the Golgi, particularly after longer treatment periods (data not shown). These data rather indicate that transport could be disturbed or endoplasmatic reticulum-assisted degradation could take place. As cell function control, we measured membrane protein sialylation, a process that requires the functioning of protein synthesis, and several complex processing and intracellular translocation steps. PCB exposure did not affect localization or abundance of glycoproteins (Fig. S1). Moreover, two inhibitors of protein processing and transport (brefeldin A, tunicamycin) did not appear to decrease Cx43p (data not shown). These results indicate that PCB treatment does not affect transport of proteins and lipids in general. But we cannot rule out that PCBs affect specifically transport of Cx43 to the membrane.

Other studies report that PCB153 does not affect the mRNA level of Cx43 but reduces the level of phosphorylated Cx43 in epithelial cells (Bager et al. 1997b; Kang et al. 1996; Simeckova et al. 2009). Phosphorylation of Cx43 is involved in its turnover and subcellular localization (Guo et al. 2003; Laird et al. 1995). Simeckova et al. (2009) suggest that PCB153 disturbs protein transport to the plasma membrane, as PCB-treated cells recovering from brefeldin A treatment take longer to restore GJIC. Moreover, they suggest that the lysosomal pathway is involved in PCB-induced degradation of Cx43. Whether these processes are also relevant for NCC, and whether they play a role for the effect of PCB on their migration, requires an extensive and meticulous follow-up study.

Are there mechanistic explanations for our observation that all ortho-PCBs reduced Cx43p, but only some inhibited GJIC? In this context, it should be noted, that our SAR for GJIC - based on 26 PCBs with varying chemical properties—differs from the two reported SARs in the literature (Hamers et al. 2011; Hemming et al. 1991). This is not entirely unexpected, as SARs for such complex endpoints like migration and GJIC may be cell-specific. For instance, some PCBs may have direct targets within GJ of all cell types, while a subgroup of PCBs may affect cell-specific pathways or protein complexes that impact then on GJIC as downstream process. Both published studies used a rat liver epithelial cell line (WB-F344) to evaluate altogether 39 PCBs. In contrast to us they found that all (except for PCB189) ortho-chlorinated PCBs inhibited GJIC (Fig. S7). When comparing SAR for different cell types, it should be considered that GJIC is only blocked if all (or most) GJ are blocked, and that the outcome of the tests depends on the sensitivity of the assay and on how the threshold for inhibition of GJIC is defined. For block of GJIC in NCC, this would mean that Cx43-based GJ, and also some others, need to be blocked. NCC express at least one other connexin, Cx45 (Fig. S10A). It is possible that some ortho-PCBs inhibit GJ of both connexins, and thus block GJIC in NCC. Other ortho-PCBs may interfere only with Cx43. This would explain why the SAR of GJ inhibition and Cx43 localization differ. Finally, it is important to consider, whether the immunopositive spots identified by our immunostain truly represented Cx43. We are convinced that the spots are indeed Cx43, because the observed localization at the membrane and in spots matches exactly with what has been reported for Cx43 (Langlois et al. 2010; Lemcke et al. 2013; Simeckova et al. 2009). Moreover, we tested two different antibodies and both resulted in the same pattern. The antibodies stained three bands at 37–40 kDa in Western blots, exactly what is reported for Cx43 (Fiorini et al. 2008; Guan and Ruch 1996; Kang et al. 1996; Matesic et al. 1994). Quantification of Cx43 levels by Western blotting showed that NIH-3T3 cells expressed high levels (consistent with earlier publications), while MDA-MB-231, HEK293 and Hela cells expressed very low levels (data not shown). These data suggest that the presence of Cx43 as such is not sufficient to allow PCB toxicity (in NIH-3T3 cells). The cells need to express the machinery regulating the re-localization of Cx43 upon PCB exposure, and they also need to express the machinery that translates the change in Cx43 distribution to an altered migration capacity. All three conditions seem to be fulfilled only in NCC.
To summarize, the present study revealed that ortho-chlorinated PCBs are strongly migration-inhibiting for human NCC and that the effect is closely correlated to a mislocalization of Cx43. To the best of our knowledge, this is the first report of migration-inhibition by non-planar PCBs. It also appears that the effect is limited to NCC or to only few cell types, as typical cell lines did not react to the tested PCBs. By testing a set of PCBs and establishing a SAR for NCC migration-inhibition, we could compare to SARs available in the literature and came up with a possible mechanism.

Notably, exploration of this comparative SAR approach for mechanistic and investigative toxicology was the main intention of this study. In this context, it should be noted that the effects observed here in vitro occur at micromolar concentrations (or marginally lower, if plastic adsorption is considered). They are thus rather not relevant for general environmental exposure of the population. Such levels would rather reflect special cases such as accidental or intentional human poisoning, or exposure of small subgroups of subjects in exceptional work situations (Pierucci et al. 2017; Ziegler et al. 2017). The mechanism identified here for such a focused scenario may apply more broadly to other toxicant classes relevant for human safety. The two NCC migration-inhibitors DDT and diehliden were found to reduce Cx43pq in other cell types (Kang et al. 1996) and are thus candidates for further investigations in our model system. Although our SAR is relatively extensive, further extensions could provide new information. For instance, PCBs with very low chlorination state have not been considered here, and neither have non-ortho chlorinated PCBs that are not dioxin-like.

A straightforward way for validation of the in vivo relevance of our study results would be comparison to the respective effects in animal models. One would imagine that such data should be available, as this group of compounds has been studied intensively. However, most data were obtained on PCB mixtures, and nearly all studies on individual compounds used planar PCBs. We could not find any in vivo study on non-planar PCBs in appropriate concentrations. It would be highly interesting to obtain such data, for example, using zebrafish, a model species in which NCC can express fluorescent proteins that allow monitoring of migration. Offspring could be analyzed for craniofacial malformations, disorders of the peripheral or enteric nervous system or behavioral abnormalities (as NCC build the adrenal medulla). If the NCC migration-inhibiting effect is as strong in vivo as observed in our in vitro experiments, the treatment could result in embryonic lethality.

A future extension of the SAR approach explored here could be to use transcriptome data, instead of published findings. Since PCBs are likely to induce many transcriptome changes, a large number of congeners would need to be tested to identify consensus changes that correlate with a phenotypic adverse effect. This way, noise could be filtered from signal in transcriptome data sets. This would probably not have been possible by use of classical transcriptomics technology, as used in one of our previous studies (Pallocca et al. 2017) due to limiting resources. However, new high-throughput transcriptomics approaches (Yeakley et al. 2017) make this approach possible, for instance in the context of the EU-ToxRisk project (Daneshian et al. 2016). Thus, the double-SAR strategy to find a mechanistic step tightly linked to NCC toxicity of PCBs, could become a general tool for identification of toxicity mechanisms.

Acknowledgements This work was supported by the Land BW, the Doreen-kamp-Zbinden foundation, the DFG (RTG1331, KoRS-CB) and the European Project EU/ToxRisk. We are grateful to M. Kapitza, H. Leisner, K. Semperowitsch, M. Brüll, the staff of the University of Konstanz bioimaging center (BIC) and the flow cytometry center (FlowKon) for invaluable experimental support. EG acknowledges support by the Fonds Nationale de la Recherche (FNR) through the National Centre of Excellence in Research (NCER) on Parkinson’s disease (IIR-BIC-PFN-15NCER). Computational analyses presented in this paper were carried out in part using the HPC facilities of the University of Luxembourg (see http://hpc.uni.lu).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.
5 Results. Project 4

Cx43 mislocalization as a key event of disturbed NCC migration

5.1 Introduction

We previously showed that PCBs disturb NCC migration and reduce the number of connexin43-positive plaques (Cx43pq) (manuscript 3). However, it is not yet clear whether reduction of Cx43pq is a causal step for the migration-inhibition. Moreover, it is not known whether connexin43 (Cx43) mislocalization might be a general pathway involved in NCC migration inhibition. To answer this question, compounds previously shown to inhibit NCC migration were tested for effects on Cx43pq number.

5.2 Materials and methods

Test compounds

All test compounds have been used previously (see manuscript 1-3). In the present study, a concentration was chosen that was close to the border of cytotoxicity. All compounds of the NTP80-list and retinoic acid (RA) were tested at the EC90V. For all other compounds, a concentration close to the border of cytotoxicity was chosen that strongly inhibited migration: cytochalasin D (CytoD): 200 nM; SP600125: 5 µM; taxol: 10 nM; brefeldin A (BFA): 500 nM; thapsigargin: 1000 nM; tunicamycin: 200 nM; CdCl2 2 µM; cytosine arabinoside (AraC, not migration inhibiting): 1 µM.

Quantification of ‘connexin43 plaques’

Quantification of Cx43pq was performed exactly as described in manuscript 3, part 2.9. Briefly, cells were cultured under standard cMINC conditions on slides with glass bottom. Treatment was applied for 3 h, before cells were fixed, permeabilized and stained for nuclei (H-33342) and Cx43. Images were acquired manually and Cx43pq quantified. For each condition, 10-15 images were analysed, averaged, and normalized to the solvent control. Data displayed are from 1-2 independent biological experiments.

5.3 Results and discussion

To investigate whether Cx43 mislocalization is a general mechanism of NCC migration-inhibitors, compounds previously shown to inhibit NCC migration were tested for their effects on Cx43pq number (Fig. 5.1). Tool compounds that interfere with the cytoskeleton (CytoD, taxol) and the JNK pathway (SP600125) did not disturb Cx43 localization. BFA, a protein transport inhibitor only had a slight effect on Cx43pq number, although the concentration used completely disassembled the Golgi (data not shown). This indicates that protein transport to the membrane is not likely the cause of the reduced Cx43pq number observed upon PCB-treatment. On the other hand, inducing ER stress using thapsigargin strongly decreased Cx43pq number. Proliferation-inhibition by AraC did not affect Cx43pq number. Compounds from the group of flame retardants, environmental and drug-like compounds were assessed. It appeared, that most organophosphorous flameretardants had no or only a weak effect on Cx43pq, but TB-BPA strongly decreased Cx43pq number. Additionally, three of six tested environmental compounds
reduced Cx43pq number, namely dieldrin, heptachlor and chlorpyriphos. Of the drug-like compounds, strong inhibition was observed for RA and valinomycin.

Organochlorine compounds (dieldrin, DDT, heptachlor, lindane) are known to interfere with Cx43pq in other cell types (Defamie et al., 2001; Jansen et al., 1996; Kang et al., 2015; Nomata et al., 1996). Interestingly, in the present study DDT did not decrease Cx43pq number, in contrast to the literature data, although GJIC was strongly decreased (data not shown). RA also strongly decreased Cx43pq. In other cell types, RA was shown to reduce the amount of phosphorylated Cx43 (Best et al., 2015; Wu et al., 2013). Moreover, the retinoid x receptor RXRα was shown to bind to a RARE sequence in the Cx43 gene and hence negatively regulates Cx43 (Gu et al., 2016). Interestingly, it is also suggested that the effect of RA is not mediated via the classical retinoid pathway (Wu et al., 2013). For organophosphorus flame retardants, TB-BPA, chlorpyriphos, hexachlorophene, diethylstilbestrol and valinomycin, no literature reports exist, to the best of our knowledge.

![Figure 5.1: Quantification of ‘connexin43 plaques’. Compounds previously shown to inhibit NCC migration were applied to NCCs for 3 h at a high but non-cytotoxic concentration. Cells were fixed, permeabilized and stained and images acquired to quantify the number of connexin43 plaques (Cx43pq). Data are expressed as means ± SD of 1-2 biological experiments. Abbreviations: AraC: cytosine arabinoside; CytoD: cytochalasin D; TB-BPA: tetrabromobisphenol A.](image)

### 5.4 Conclusions

These preliminary results suggest that Cx43 mislocalization might indeed be a mechanism of NCC migration-inhibition shared by several toxicants. In particular, organochlorinated compounds interfered with Cx43pq number.

Comparison of the Cx43pq results with cell speed measurements further revealed, that Cx43 mislocalization is not correlated with reduced cell speed: DDT reduces cell speed but not Cx43pq, whereas chlorpyriphos and dieldrin do not reduce cell speed but Cx43pq. Heptachlor and RA affected both endpoints. These results suggest that different compounds affect different biological processes that subsequently lead to migration-inhibition. In a next step, it is important to test more unspecific NCC toxicants to investigate whether Cx43 mislocalization is unique to NCC migration-inhibiting compounds.
6 Discussion

6.1 Development of a high-throughput NCC migration assay

NCC are a unique fetal cell type and disturbance of NCC function can lead to neurocristopathies. In the past, effects of toxicants on NCC function was mainly assessed using the low-throughput technology of neural tube explants of model animals. With the development of hESC technology, human NCC became available and a first human cell-based NCC migration assay was established (Dreser et al., 2015; Zimmer et al., 2012, 2014). The aim of the present work was to adapt this assay for high-throughput applications.

6.1.1 Comparison of the MINC and cMINC assays

Towards a high-throughput assay

The main goal of the assay adaptation was to make it experimenter-independent and to enable automated image acquisition (Fig. 6.1). Introducing a cell-free area by scratching requires a skilled operator and training. In contrast, in the cMINC assay reusable silicon stoppers are used. In the later case, the cell-free area is of reproducible size within and among experiments, which also decreases variation. Moreover, in the cMINC assay, the cell-free area is always at the same position, which allows automated image acquisition. In contrast to this, the MINC assay required manual image acquisition by a trained experimenter, which limits throughput and is sensitive to experimenter bias.

<table>
<thead>
<tr>
<th></th>
<th>MINC</th>
<th>cMINC</th>
<th>Advantage of cMINC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well format</td>
<td>48-well</td>
<td>96-well</td>
<td>Saves material</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Faster (automated) coating</td>
</tr>
<tr>
<td>Seeding density</td>
<td>50’000/well (67’000/cm²)</td>
<td>30’000/well (95’000/cm²)</td>
<td>Reducing time before migration</td>
</tr>
<tr>
<td>Time before migration</td>
<td>48 h</td>
<td>24 h</td>
<td>Less inter-experiment variation due to differences in proliferation among batches</td>
</tr>
<tr>
<td>Toxicant treatment</td>
<td>48 h</td>
<td>24 h</td>
<td>Reducing proliferation effects of toxicants</td>
</tr>
<tr>
<td>Total assay duration</td>
<td>5 days</td>
<td>4 days</td>
<td>Saves time</td>
</tr>
<tr>
<td>Cell-free area</td>
<td>Scratch</td>
<td>Silicon stoppers</td>
<td>Constant size = less variation</td>
</tr>
<tr>
<td>Viability measurement</td>
<td>Resazurin</td>
<td>Cell count</td>
<td>Less intra-experiment variation</td>
</tr>
<tr>
<td>Image acquisition</td>
<td>Manual</td>
<td>Automated</td>
<td>Higher throughput</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No experimenter-bias</td>
</tr>
<tr>
<td>Skill level</td>
<td>Intensive training</td>
<td>Simple (1 week)</td>
<td>Experimenter-independent</td>
</tr>
</tbody>
</table>

Figure 6.1: Comparison of the assays. The new cMINC assay is compared to the established MINC assay (scratch assay) as previously described (Dreser et al., 2015; Zimmer et al., 2012, 2014).

Other differences

Some smaller changes were also introduced. The well format was changed from 48-well to 96-well, reducing material consumption. Cell seeding density was slightly increased to start migration one day earlier, limiting inter-experimental variation in cell count due to proliferation effects. More importantly, the toxicant treatment was reduced from 48 h to 24 h, reducing effects of proliferation-inhibiting test compounds. In the MINC assay, viability was measured by resazurin reduction, e.g. metabolic activity. In
the cMINC assay, viability is measured as cell count. Taken together, the new cMINC assay has several advantages that all together facilitate high-throughput measurements.

6.1.2 Main features and challenges of the cMINC assay

Finding a window of sensitivity
In a first step of assay optimization, the cMINC assay was run with 48 h exposure time, identical to the MINC assay. However, in the cMINC setup, 48 h treatment did not result in a wide enough ‘window of sensitivity’. Known NCC migration-inhibitors (i.e. CdCl$_2$, As$_2$O$_3$) mainly affected migration at concentrations that already reduced viability. To overcome this problem, exposure time was reduced to 24 h with the rational that the compounds would still have enough time to reduce migration but less time to act cytotoxic or to reduce proliferation. Indeed, the 24 h proved to have a similar ‘window of sensitivity’ than the MINC assay.

Proliferation as confounding factor
However, proliferation is also in the 24 h setup still of concern. For this reason, a variation of the cMINC assay was developed that allowed to measure proliferation in the same assay format. The experimental results (Manuscript 1, Fig. 6) revealed that many compounds positive in the cMINC assay also affect NCC proliferation. Therefore, cMINC ‘positive hits’ should be carefully evaluated for effects on proliferation.

Assay setup with positive and negative control compounds
The problem with the ‘window of sensitivity’ also shows, that it is of utmost importance to carefully establish an assay with appropriate positive and negative controls (Aschner et al., 2016; Kadereit et al., 2012). In the present assay, two kinds of positive controls were used: Compounds known to interfere with migration in other cell types due to interference with the migration machinery (microtubuli, actin skeleton, ...) and compounds known to be toxic to NCC (CdCl$_2$, As$_2$O$_3$).

Establishing a preliminary prediction model
As the cMINC assay has two endpoints (migration and viability), the prediction model has to take into account both of them. The use of appropriate control compounds helped to establish a prediction model. Especially ‘unspecific cytotoxicants’ were useful to define a threshold below which migration-inhibition was considered not specifically affected. However, compounds exceeding this threshold do not necessarily need to be D(N)T toxicants. At present it is not known how the in vitro cMINC results would translate to expected in vivo results. Therefore, the prediction model has to be considered as preliminary.

6.1.3 Development status of the cMINC assay

Last year, the OECD assessed all existing DNT assays for readiness to include in a prospective DNT test battery (OECD 2016). As the NCC migration assay is a candidate to be included, readiness of the new cMINC is of interest. Therefore, the evaluation parameters were updated to the cMINC assay (Fig. 6.2). Overall, the cMINC assay performs well in many evaluation parameters. However, some important criteria are not yet met. At present, the assay lacks evaluation of assay specificity, sensitivity and accuracy. Moreover, between-laboratory reproducibility has not yet been tested. This is also hindered by the use of
6.1 Development of a high-throughput NCC migration assay

hESC-derived NCC, as culturing of hESC needs ethical approval. Additionally, maintenance of hESC and differentiation of NCC requires a lot of expertise.

<table>
<thead>
<tr>
<th>Evaluation parameter</th>
<th>Readiness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Biological Plausibility</td>
<td>NCC migration is essential for embryonic development. ++</td>
</tr>
<tr>
<td>2. Extrapolation to humans</td>
<td>Use of hESC-derived cells. ++</td>
</tr>
<tr>
<td>3. Availability of Resources</td>
<td>Use of hESC is restricted. - -</td>
</tr>
<tr>
<td>5. Within-laboratory reproducibility</td>
<td>yes, also among different experimenters ++</td>
</tr>
<tr>
<td>6. Between-laboratory reproducibility</td>
<td>not assessed - -</td>
</tr>
<tr>
<td>7. Assay variability</td>
<td>Viability: &lt;10%. Migration: approx. 15%. ++</td>
</tr>
<tr>
<td>8. Accuracy</td>
<td>not assessed - -</td>
</tr>
<tr>
<td>9. Assay specificity</td>
<td>not assessed, but questionable -</td>
</tr>
<tr>
<td>10. Assay sensitivity</td>
<td>not assessed, but rather high +</td>
</tr>
<tr>
<td>11. Dynamic range</td>
<td>Migration distance from 10-150%. ++</td>
</tr>
<tr>
<td>12. Concentration test range</td>
<td>nM – mM depending on the compound. Use of 2-fold dilution steps. ++</td>
</tr>
<tr>
<td>13. Detection/Adjustment of confounding factor and/or incorrect/inconclusive measurements and/or other bias</td>
<td>Cytotoxicity measured within the assay. ++</td>
</tr>
<tr>
<td>14. Response Characterization</td>
<td>Migration-inhibition ≥25% and Viability ≥90%. +</td>
</tr>
<tr>
<td>15. Technological Transferability/Proprietary elements</td>
<td>Restriction for use of hESC. Differentiation of NCC requires expertise. - -</td>
</tr>
<tr>
<td>17. Documentation</td>
<td>not available - -</td>
</tr>
</tbody>
</table>

Figure 6.2: Readiness criteria of the cMINC assay. Readiness of the cMINC assay according to the evaluation parameter defined in OECD [2016]. Readiness is indicated as ++: criterion fully met, +: criterion partially met, -: criterion partially not met, – –: criterion not met.

6.1.4 Current limitations and possible solutions

Assay validation and cell characterization

Before use of the cMINC in a test battery, assay validation should be performed. However, this is difficult as ‘gold standard’ toxicants for NCC migration-inhibition are scarce. It would also be desirable to characterize the NCCs more. At present it is not known to which developmental stage and position along the body axis the generated NCC would correspond. This is relevant, because animal experiments have indicated different behaviours of cranial and trunk NCC towards toxicants (Dunn et al. 1995; Usami et al. 2014).
Production of NCC is limiting
As mentioned above, an important limitation is the NCC production. This problem could be overcome
by (1) the use of iPSC-derived NCC, and (2) simplification of the NCC differentiation protocol. Both
approaches have successfully been tested in proof-of-concept experiments (Manuscript 1, Fig. S2) and
should be further developed.

High-throughput considerations
It should also be noted that the assay is restricted for downscaling. At present, 96-well format is used,
but the use of 384-well plates might be possible (Joy et al. 2014). Further downscaling is probably
not possible. The well diameter of 1536-well plates is roughly 1.5 mm, comparing to a NCC migration
distance of 1 mm/day, this might be too small. However, several plate handling steps could be executed
by pipetting robots, which is favorable for high-throughput measurements. Furthermore, commercially
available 384-well plates (www.platypustech.com) would eliminate the necessity to introduce and remove
the silicon stoppers, the two steps that are difficult to automate otherwise.

6.2 Use of the cMINC assay for screening purposes

6.2.1 Screening strategy

Tiered strategy and single concentration testing
The screening of the 80-compound library was performed in a tiered procedure. In a first step, the NOAEL
was determined and in a next step migration at this concentration was measured. Only for 'potential
positive hits' concentration-response curves were obtained and follow-up experiments performed. This
tiered strategy was chosen because (1) the used NCCs are precious and (2) many screen compounds
were expected to be non-cytotoxic at the highest test concentration (based on unpublished data).
This strategy has proven useful. Indeed, only 12/80 compounds were cytotoxic. A majority of test
compounds (50/80) did not disturb migration at the highest test concentration and were waived from
further testing. Testing concentration ranges for these compounds would not have added any value but
would have wasted resources.

Screening and hit-confirmation
The screening resulted in 23 confirmed 'positive hits' among the 75 unique compounds (e.g. 31%). This
number is comparable to the hit rate in a previous screen using the MINC assay where 11/28 (39%)
compounds disturbed migration (Zimmer et al. 2014). However, the hit rate is higher than in published
and unpublished studies testing for neurite outgrowth using the same library where only 10-15% were
hits (Ryan et al. 2016 Delp and Klima, personal communications). These results suggest that on the
one hand, the NCC migration assay has a high sensitivity (e.g. picks up most 'true positives'), but on
the other hand, maybe to the expense of specificity (e.g. wrongly picks up 'negatives').
In the hit-confirmation phase, 23/26 compounds were confirmed. This indicates on the one hand, that
the assay works reliable and reproducible but on the other hand, that the hit-confirmation phase is also
important to eliminate false positives. For the screening part, the threshold for potential hit compounds
was deliberately chosen less stringent than under standard cMINC conditions (migration <80% vs. <75% and viability >85% vs. >90%). This bears the risk to increase the false positive rate. However, the hit confirmation showed that (1) positive compounds (e.g. chlorpyriphos, dieldrin) would have missed otherwise and (2) the three non-confirmed compounds would have also been picked up using the more stringent thresholds.

Future directions
All together, the tiered approach and the less stringent thresholds during screening were useful. The hit-confirmation phase is a necessary step to eliminate false positives. However, it might be possible to further save resources by decreasing the amount of biological or technical replicates. For example during the screening, compounds that obtained clear negative results in two biological experiments could be waived from the third experiment. Also, the concentration-response curves of the hit-confirmation could maybe be reduced to two biological experiments (with 4-5 technical replicates) or three biological experiments (with i.e. 3 technical replicates). Most importantly, the sensitivity and specificity of the assay should be evaluated.

6.2.2 Review of the prediction model
One reason why the assay has a high sensitivity might be a too loose prediction model. For the screening project (manuscript 2), hit definition was based on an efficacy (% migration-inhibition) of 25%. However, assay development and calibration with unspecific and specific toxicants revealed, that compounds believed to be unspecific can reduce migration by as much as 30% (i.e. Triton-X 100, manuscript 1). On the other hand, there are also compounds that are believed to be migration-inhibitors (based on cell speed reduction) in this zone (i.e. carbaryl, PBDE-99).

Representation of all tested compounds according to their specificity and efficacy reveals, that they cover a large space (Fig. 6.3). One group of compounds has an efficacy < 25% and (by definition) a specificity < 1 (Fig. 6.3 green). These are considered as non migration-inhibitors. Among them are compounds that affect the migration readout by inhibiting proliferation (i.e. AraC, aphidicolin). Another group consists of compounds with a large efficacy (> 40%) and/or a large specificity (> 3) (Fig. 6.3 red). To this group belong ortho-chlorinated PCBs, DDT, taxol and RA. Many - but not all - compounds of this group were shown to reduce also single cell speed. Even if single cell speed is not disturbed, a migration-inhibition of >40% is likely to be biologically relevant in vivo. Hence, this group of compounds should be considered as strong NCC migration-inhibitors. Between these two zones, there is an intermediate zone of compounds with efficacy > 25% (Fig. 6.3 orange). Especially in the zone of relatively low efficacy and a ratio close to 1, it is unclear if compounds should be considered as migration-inhibitors.

Until there is further evidence, I would suggest to revise the prediction model and introduce three categories: 'strong migration-inhibitors', 'weak migration-inhibitors' and 'no alert'. Especially the group of weak migration-inhibitors needs further attention. For example, in follow-up experiments as suggested in manuscript 2.
6.3 Compounds disturbing human NCC migration

The cMINC assay was challenged on the one hand with compounds of known mode of action and on the other hand with environmental compounds and other chemicals of interest.

Compounds with known mode of action

A broad set of compounds with known mode of action was tested in the cMINC assay to obtain information on which pathways are relevant for NCC migration and could be potential toxicological targets (Fig. 6.4). Most compounds interfering with the cytoskeleton disturbed NCC migration. This is not surprising, but only shows proof-of-concept. Proliferation-inhibition does not necessarily affect migration, nor do inflammatory processes play an important role. In contrast to this, protein synthesis and protein transport, but not protein degradation, seem to be important for NCC migration. Moreover, gap junctions could play a role, as both inhibitors disturbed NCC migration. Agonists and antagonists of several receptor
systems were also tested. Mostly, interventions on the RA receptor systems disturbed migration, but other receptor systems do not seem to play an important role. In a last step, various cytokines were tested, many of them were shown to affect cell migration in other cellular systems. However, NCC did not respond to them, except for PDGF-AA that slightly increased migration (data not shown).

To summarize, these results show that (1) the system reacts to known migration-inhibitors and (2) is not prone to very unspecific influences. The fact that RA receptor agonists and antagonists both disturbed NCC migration suggests that compounds known to interfere with this system should be prioritized for cMINC testing. Future studies could also investigate whether synthesis of a particular protein is necessary for NCC migration or whether migration is disturbed by general cellular stress.

### Figure 6.4: Tested compounds part I: Compounds with defined mode of action.

Compounds are grouped in columns according to the updated prediction model. The molecular target is indicated in brackets. The indicated concentration is the highest tested concentration for compounds that were not cytotoxic.
### Discussion

#### Persistent Organic Pollutant

<table>
<thead>
<tr>
<th>Strong</th>
<th>Weak</th>
<th>No Alert</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT</td>
<td>Triclosan</td>
<td>2,3,7,8-TCDD 75 nM</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>Lindane</td>
<td>20 µM</td>
</tr>
<tr>
<td>Heptachlor</td>
<td></td>
<td>dioxin-like PCB (3 tested, see ms 3)</td>
</tr>
<tr>
<td>Hexachloropane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ortho-chlorinated PCB (25 tested, see ms 3)</td>
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#### Organobromines

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<tr>
<td>Dioxin-like PCB</td>
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#### Organophosphates

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<tr>
<th>BPDP</th>
<th>ETA-DOPO</th>
<th>DOPO 100 µM</th>
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<tr>
<td>EDA-DOPO</td>
<td>Tricresyl phosphate</td>
<td>EMIM DEP 20 µM</td>
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<td>Tris(2-chloroethyl) phosphate</td>
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#### Other pesticides

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<th>Triazoles</th>
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#### Other environmental compounds

<table>
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<tr>
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</tr>
<tr>
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<td>CdCl2</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Other industrial</td>
<td>Acrylamid</td>
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<td>Various compounds</td>
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</tr>
<tr>
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<td>Triton X-100</td>
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**Figure 6.5: Tested compounds part II: Environmental, drug-like and other toxicants.** Compounds are grouped in columns according to the updated prediction model. The indicated concentration is the highest tested concentration for compounds that were not cytotoxic. Abbreviations: EMIM DEP: 1-Ethyl-3-methylimidazolium diethylphosphate; IPBC: 3-Iodo-2-propynyl butylcarbamate; MMT: Methylcyclopentadienyl manganese tricarbonyl; TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin.
Environmental and drug-like compounds
Testing of several classes of environmental compounds, industrial chemicals and drug-like compounds (Fig. 6.5) revealed, that many halogenated organic and organophosphorous compounds are strong NCC migration-inhibitors. Several metal-containing compounds are weak NCC migration-inhibitors, whereas the majority of non-POP pesticides, industrial and drug-like compounds as well as all PAH were not toxic to NCC at the tested concentrations.

Flame retardants from several chemical classes (PBDE, organophosphates, DOPO-derivates), e.g. structurally not closely related compounds, were tested. Most of them disturbed NCC migration, but at different concentrations. For example, DOPO-derived flame retardants were toxic at 25-100 µM, whereas organophosphorous and polybrominated flame retardants were toxic at 5-10 µM. If they have the same flame retarding capacity, this would indicate that DOPO-derived flame retardants are less hazardous to NCC. Interestingly, there are some specific chemical structures that seem to be toxic to NCC: Only organophosphorous flame retardants with aromatic side chains, but not aliphatic ones, and only non-planar, but not dioxin-like PCBs did interfere with NCC migration.

To summarize, these results indicate that the cMINC assay might be more suitable to prioritize environmental chemicals than drug-like compounds.

Comparison with NCC toxicants from literature
Only few compounds have been described in the literature as NCC toxicants (see part 1.3.4). They comprise ethanol, cyclopamine, RA, triazole-derived fungicides and VPA. Of these five, only the last three compounds have been tested in the cMINC assay.

RA strongly inhibited NCC migration and reduced cell speed. Also related compounds like retinaldehyde and RA receptor antagonists disturbed NCC migration. Several triazole-derived fungicides were tested in the cMINC assay (Fig. 6.5), but only triadimefon reproducibly disturbed NCC migration.

VPA had only a weak effect in the cMINC assay. It should be noted that for VPA only one ex vivo study found an effect using chicken neural tube explants (Fuller et al., 2002). In this study, the apparent inhibition of migration could also be due to inhibition of proliferation. Moreover, another study using rat neural tube explants could not confirm an effect on NCC migration (Usami et al., 2015).

Comparison of MINC and cMINC hits
With the previously established MINC assay, approximately 50 compounds have been tested for NCC migration-inhibition. Although the cMINC assay was performed in the same lab with cells from the same differentiation protocol, results could only partially be reproduced (Fig. 6.6). Surprisingly, some compounds were only effective at 10 times higher concentrations compared to the MINC assay. It could be that some compounds were not reproduced because the cMINC assay more strictly controls for cell proliferation and cell viability. Moreover, it might be that some compounds are only effective upon long (48 h) exposure but not in the shorter scenario.
Discussion

Figure 6.6: Comparison of MINC and cMINC hits. Hit compounds from the MINC assay (Dreser et al., 2015; Zimmer et al., 2012, 2014) were compared to results of the cMINC assay.

Future directions

As it appeared that NCC are particularly sensitive to halogenated and organophosphorus compounds, it will be of interest to find the mechanism. Moreover, in vitro studies with non-mammalian or mammalian models could help to answer the question whether this toxic effect is relevant in vivo. However, information from risk assessment and exposure data should be taken into account (Fig. 6.7). Many NCC toxicants were effective only at relatively high concentrations (10-20 µM) and it is questionable whether such concentrations would be reached in vivo. On the other hand, it is at present also not known which fraction of the toxicants stick to the culture dish and are thus not available for the NCC. Therefore, physiologically based pharmacokinetic (PBPK) modelling would help to estimate the corresponding in vivo concentration that is expected to induce NCC toxicity.

<table>
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<th>Reproduced at higher concentration</th>
<th>Not reproduced</th>
</tr>
</thead>
<tbody>
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<td>Triadimenol</td>
</tr>
<tr>
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<td>PCB170 (0.5 vs 5 µM)</td>
<td>Lead acetate</td>
</tr>
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<td>PCB180</td>
<td>VPA (1 vs 10 mM)</td>
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<td>PBDE-99</td>
<td>CdCl₂ (100 vs 1000 nM)</td>
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<td>As₂O₃</td>
<td>Rotenone (1 vs 30 nM)</td>
<td>Gleevec</td>
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<td>Methoxyacetic acid</td>
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<td></td>
<td>TSA</td>
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6.4 Testing strategy considerations

6.4.1 Testing strategy of NCC toxicity assessment

NCC toxicity assessment in a tiered strategy

As outlined above, the tiered testing strategy for the cMINC assay has proven useful. One could even go one step further to reduce use of the expensive NCCs (Fig. 6.8): A viability assay could be run in a small assay format (384 or 1536-well format) and with few replicates (2 technical replicates, 1-3 biological replicates). In a next step, compounds would be tested in the cMINC assay at the EC90V. Based on the amount of migration-inhibition at the EC90V, compounds would be classified as negative or weak/strong positives. Positive compounds would then be tested in more detail, as suggested and applied in manuscript 2. Additionally, one could envisage to develop an NCC proliferation assay, as proliferation...
is another important NCC function and known to be a target of teratogens (see part 1.3.3). Such a proliferation assay could for example consist of an EdU incorporation assay, as used in manuscript 1 and 2. The assay could be performed at high throughput, in a small well format and few replicates. The combined data from viability, proliferation and migration assays could then be combined to a single ‘NCC toxicity estimate’ and integrated with other data (other D(N)T assays, risk assessment, ...).

**Figure 6.8: Suggested testing strategy to assess NCC toxicity.** This tiered approach consists of (1) a viability assay, (2) the cMINC assay, (3) follow-up assays and could be complemented with (4) a proliferation assay.

**Role of follow-up migration assays**

Results of manuscript 2 showed, that follow-up assays can give valuable, complementary information. It is therefore strongly suggested to include them in a testing strategy. On the one hand the follow-up assays serve as a confirmation step of compounds. On the other hand, they also provide information about possible mechanisms. For example, a compound that reduces cell speed of all cells by 50% will lead to a reduction of 50% in the cell tracking assay, but would not affect transwell migration (Fig. 6.9). On the other hand, a compound that immobilises 50% of the cells would be detected in the transwell assay but would have little effect on the measured cell speed. Results of manuscript 2 have shown, that many toxicants rather act via the second mechanism. Only few toxicants reduced cell speed, but these toxicants then had profound effects on the amount of migrated cells.

In the future, one could also consider to include the follow-up assays in the prediction model. For example, ‘weak migration-inhibitors’ could be reclassified as ‘strong’ if they substantially decrease cell speed. Or ‘weak’ compounds that can not be confirmed in follow-up assays could be reclassified as ‘no alert’.

**Figure 6.9: Different migration assays measure different biological processes.** Three different situations are shown: normal or untreated condition as well as two different, hypothetical toxicants. The colored circles represent NCCs, the length of the arrow indicates the speed of each cell. Expected results of the three assays are stated below.
6 Discussion

6.4.2 Position of NCC function assays in the Integrated Testing Strategy

Based on the limitations outlined in 6.1.4, the cMINC assay can only reach a medium throughput. However, it could be part of a test battery for D(N)T testing (see part 1.2.4, Fig. 1.3). Most of the suggest D(N)T assays have at present a limited throughput (OECD, 2016). The assay with the highest throughput is currently the neurite outgrowth assay. The cMINC assay has a lower throughput, but still higher than most other suggested D(N)T assays.

The D(N)T test battery could be placed downstream of high-throughput screening assays (Fig. 6.10), i.e. the existing ToxCast assays and assays testing for interference with nuclear receptors (Judson et al., 2015; Lynch et al., 2017). Compounds with suspected D(N)T potential would be prioritized to run through the D(N)T test battery. A D(N)T potential would be suspected for example if the compounds interfere with thyroid hormone receptors or - particularly with respect to NCC migration - RA receptors.

Data from the test battery should then be combined to estimate the D(N)T potential. Based on these results, compounds can be prioritized for down-stream assays. Such assays can include in vivo testing in non-mammalian animals or more complex in vitro mechanistic studies.

Relevance of NCC toxicity information

As the cMINC assay needs costly cell production and is limited in downscaling one might ask whether it is worth at all to perform the cMINC assay. The assay should only be used in a test battery, if it gives additional information, e.g. information that would not be obtained from other (higher throughput, cheaper) test systems. At present, the amount of data to answer this question is scarce. First results come from the comparison of the NTP80-compounds among different test systems. This compound list has been tested besides the cMINC assay in three neurite outgrowth assays and a cardiotoxicity assay (Ryan et al., 2016; Sirenko et al., 2017; Delp and Klima, unpublished data). Comparison of the ‘specific’ hits of each test system reveals, that the cMINC assay shares more hits with the neurite outgrowth assays than with the cardiotoxicity assay (Fig. 6.11). Moreover, most neurite outgrowth hits were also cMINC hits, but not vice versa. This indicates that the cMINC assay has the potential to detect compounds that would not be detected in the high-throughput neurite outgrowth assay. In particular it is interesting to note, that many drug-like cMINC hits are shared with the neurite outgrowth assay, but that two pesticides and several flame retardants were unique to the cMINC assay. The drug-like hits had in the cMINC assay rather a low specificity and efficacy, which could indicate that some effects could be due to a more broad toxicity mechanism, may be also related to the onset of cytotoxicity. On the other hand, many of the unique cMINC hits had a high specificity, which indicates that the compound might target a
cellular process important for NCC.

To summarize, these preliminary results indicate that the cMINC assay might indeed give additional information and detects compounds not recognized by other assays. However, at present only data from five assays with 80 compounds is available. Future studies using more compounds and comparing more different test systems should be used to shed light on this question.

**Figure 6.11:** Overlap of the (specific) hit compounds of the cMINC with other cell function assays. (A) All compounds of the NTP80-list that were specific in at least one assay are listed. NCC migration results are compared with data for cardiotoxicity (Sirenko et al., 2017) and the combined data of three neurite outgrowth assays using GABAergic/glutamatergic, dopaminergic and peripheral neurons (Ryan et al., 2016, Delp and Klima, unpublished data). Compounds positive in the cMINC assay are highlighted in bold. Color code: green: flame retardants; blue: pesticides; pink: drug-like compounds; brown: industrial; yellow: PAH; gray: negative controls. (B) Overview of the overlap of hit compounds of the cMINC assay with both other assay types. The numbers indicate the number of shared and unique ‘specific’ hit compounds of the assays.
6.4.3 Translation of in vitro results to the in vivo situation

A basic assumption for the relevance of the cMINC data is, that there is a "critical mass" of NCCs that need to arrive at the target tissue. If cells migrate slower and therefore fewer cells arrive at the target, this is assumed to affect NCC function. But as the cells are also at the same time proliferating, inhibition of proliferation can also reduce the critical NCC mass.

At present, we do not know whether reducing cell speed and reducing the number of migrating cells has the same effect. The reduction of cell number is similar to Treacher-Collins syndrome, whereas reduced cell speed is similar to Hirschsprung’s disease. In both cases, disturbed NCC function results in tremendous developmental defects.

What is a biological meaningful effect level?

At present, it remains unanswered to which extend migration-inhibition has to occur in the in vitro assay to result in in vivo disturbances. A 10% reduction in migration could already have drastic consequences, or a >50% loss of migration could remain without consequences, if there are compensatory mechanisms.

Differences to the in vivo situation

An in vitro assay is inevitably a simplified model of the in vivo situation. For example, NCCs migrate in a three dimensional environment in vivo, whereas in the cMINC assay cells grow in a two dimensional culture. In vivo, NCCs migrate in chains, streams or as single cells, and the migration type depends on the localization, timing and species (see part 1.3.2). In the cMINC assay, most cells migrate as single cells, sometimes they also form streams. From this can be concluded, that the assay is limited and would not be suitable to detect toxicants that affect the group or three dimensional migration behavior.

Lastly, in vivo there are gradients of signaling molecules in the fetus and NCC react to such gradients. As such gradients are not present in cell culture, this indicates - as illustrated in Fig. 9 in manuscript 2 - that the cMINC assay is not suited to measure polarity and sensing events.

To summarize, a strong migration-inhibition in vitro is likely to translate to an observable phenotype in vivo, if the tested concentrations are achieved in vivo. However, in vitro assays are a simplification of the real world and therefore we cannot expect an in vitro assay to be fully predictive of the in vivo situation.
6.5 Finding mechanisms of migration-inhibition

One of the main features of the cMINC assay is that it is a functional D(N)T assays. However, this is also one of the main drawbacks: the assays does not give information on the mechanism of migration inhibition. Time-lapse video microscopy, performed for the follow-up studies, gave at least some first hints: Only some migration-inhibiting compounds reduce cell speed drastically. For other compounds, visual inspection of the time-lapse video did not reveal any gross changes in migration behaviour of the cells. Rather, it appeared that some compounds affect migration on a population level by changing the number of cells migrating into the cell-free area (unpublished observation).

Possible mechanisms of migration-inhibition

Toxicants could interfere with cell migration by affecting several, unrelated cell functions. Typical cell functions involved in migration are remodeling of the cytoskeleton, adhesion, directionality and persistance of migration (see also Fig. 9 in manuscript 2). Also perception of cell-cell and cell-environment signals are important. But also energy supply can be disturbed or toxicants can induce a shift in cell fate, for example by inducing differentiation.

At present, we only have scarce information about possible mechanisms. Clearly, compounds disturbing cytoskeletal remodeling (i.e. CytoD, taxol) interfere with migration. Interestingly, also compounds inducing ER stress (i.e. tunicamycin, thapsigargin, brefeldin A) strongly inhibited migration (see. Fig. 6.3). There are also indications for involvement of mitochondrial function, as rotenone and valinomycin were cMINC hits. Lastly, interference with localization or non-channel functions of Cx43 could be a mechanistic step.

Strategies to find mechanisms

In the course of this thesis, several strategies were used to obtain mechanistic information. A simple technique that allows to test several pathways easily is gene expression studies by qPCR or transcriptomics. Gene expression of several pathways was tested, i.e. cell adhesion molecules, nuclear receptors and differentiation markers. However, the tested toxicants did not induce strong gene expression changes or the observed changes were not relevant for the migration inhibition (i.e. CYP1A induction by non-ortho PCBs). Also, previous studies of NCC transcriptome changes upon toxicant treatment did not identify common toxicity pathways. (Pallocca et al. 2016 2017).

Immunocytochemical staining can be used for few candidate KE, i.e. disruption of the cytoskeleton can be assessed. This only works for non-secreted proteins where good antibodies exist. More successful was the strategy to test different agonists and antagonists of signaling pathways, although it was rather useful to exclude pathways than to find the particular KE.

The most successful approach was to screen the literature for possible mechanisms and compare it to mechanisms important for NCC migration. By this way, gap junctional communication was identified as a candidate KE. In the course of the study, it turned out that GJIC is not a causal event, but that distribution of the gap junctional protein Cx43 was correlated to migration-inhibition in a set of PCBs.
Disturbance in Cx43 localization as a key event

The results in manuscript 3 revealed that all PCBs that interfere with NCC migration also reduce the number of Cx43pq. This indicates that Cx43pq could be a KE in PCB-induced migration-inhibition. There is evidence in the literature that reduction of Cx43 by knock-out or knock-down causes disturbed NCC migration. However, it should also be noted that at present we do not have direct evidence that the reduction in Cx43pq is causal for the observed NCC migration-inhibition in our experiments. It is inevitable to perform Cx43 knock-down experiments using the hPSC-derived NCC and closely monitor if Cx43 localization correlates with the migration phenotype.

Another strategy to strengthen the hypothesis of a causal role of Cx43 was to test other NCC migration-inhibiting compounds (see chapter 5). Preliminary experiments were performed using a set of chemically diverse migration-inhibitors (Fig. 6.12). Several of the tested compounds clearly affected Cx43pq number, namely TB-BPA, heptachlor, dieldrin and chlorpyriphos as well as RA and valinomycin. In particular the last two compounds are of special interest. RA is a known NCC teratogen, and could induce teratogenicity by mis-regulating the RA system. However, the actual results add Cx43 mislocalization to the list of potential mechanisms. Valinomycin is a compound that is known to act on mitochondria, usually at lower concentrations than those effective in the cMINC assay. Therefore, mitochondrial disruption is rather unlikely to be causal for NCC toxicity. Therefore, the observed Cx43 mislocalization adds valuable information.

In a next step, it is essential to test a broader set of compounds for interference with Cx43 localization. In particular, several non-migration-inhibiting compounds should be tested to rule out that Cx43 mislocalization is a frequent event induced by many compounds but not related to migration-inhibition.
6.6 Conclusion

Aim 1: Assay development
In the course of this thesis, a NCC migration assay suitable for high-throughput was set up. Compared to the existing assay (MINC), the new assay is experimenter-independent and allows automated image acquisition, which results in a higher throughput and more reproducible results. Several positive and negative control compounds were used to assess assay performance. Proliferation was identified as a potential confounding factor, its impact was assessed and strategies to circumvent the problem were developed. Finally, a preliminary prediction model was established.

Aim 2: Screening of a compound library
Using this high-throughput assay, an 80-compound library was successfully screened. The applied tiered strategy was useful and allowed to detect the migration-inhibiting compounds in an economical manner. The screening identified several NCC migration-inhibiting compounds from the chemical classes of flame retardants, pesticides and drug-like compounds. In particular the environmental compounds were stronger NCC migration-inhibitors than the drug-like compounds and indicate, that the assay might be suitable rather to prioritize environmental compounds than drug-like substances. The screening also revealed that NCCs are particularly sensitive to halogenated or phosphorous organic compounds and that some of these compounds were not toxic in screening assays using other cells. Moreover, three follow-up assays were developed and all ‘positive hits’ were subsequently tested. These assay delivered valuable, complementary information. For example it revealed, that only some compounds interfere with NCC migration by affecting single cell speed.

Aim 3: Identification of a potential mechanism
The known NCC migration-inhibiting effect of PCBs was confirmed using three different migration assays. Various potential mechanisms were excluded based on interventions with agonists/antagonists or gene expression studies. Subsequently, a structure-activity relationship was established and compared to published data. Interference with GJIC was a starting point for future investigations and lead to the identification of Cx43 mislocalization as a potential KE. Importantly, all migration-inhibiting PCBs disturbed Cx43pq number, whereas non-toxic PCBs did not affect Cx43pq. Based on the available literature, Cx43 mislocalization could be causal for the observed migration-inhibition, i.e. by interference with microtubule stability and directionality. Moreover, Cx43 is highly expressed in NCC and known to be important for NCC migration. However, at present, there are no direct proofs that Cx43 mislocalization causes the observed migration-inhibition.

Aim 4: Relevance of this mechanism
In preliminary experiments, several NCC migration-inhibiting compounds were tested for interference with Cx43 localization. A few compounds from different chemical classes reduced Cx43pq number. This points to the fact that Cx43 mislocalization could indeed be a KE tightly linked to migration-inhibition, although more investigations are needed. All in all, the strategy to identify a potential mechanism using one compound class was successful.
6.7 Outlook

Further development of the assay
At present, the throughput of the assay is partially limited by the laborious production of NCCs. First steps towards a shorter differentiation protocol and the use of iPSC-derived NCCs have already been undertaken. Moreover, the use of a smaller well-format (384-well) would be favorable. It would be desirable to further characterize the NCCs. Although presence of NCC marker has been tested, it would be of interest to know the differential potential of the cells and to which axial levels the cells correspond.

Before high-throughput screening are initiated, specificity, sensitivity and accuracy should be thoroughly tested and the between-laboratory reproducibility assessed. Additionally, the prediction model should be validated and, if necessary, updated.

Biological relevance and test strategy
For revision of the prediction model, it would be of great value to have more in vivo data of compounds toxic to NCC. Vice-versa, also in vivo data of cMINC hits would be of interest, as at present it is not clear if the cMINC hits are relevant in vivo. For this purpose, studies with non-mammalian animals could be a first step. For example, using zebra fish with labeled NCCs would reveal whether certain toxicants interfere with NCC migration also under in vivo, three-dimensional conditions. Observation of structural deficits like craniofacial or heart malformations could also indicate a NCC defect.

To optimize the overall test strategy, it is necessary to test the same compounds used in the cMINC assay also in other high-throughput assays. This will give information whether the NCC assay delivers complementary information. A first comparison with four other assays was promising (Fig. 6.11).

Finding mechanisms of migration-inhibition
Mislocalization of Cx43 was identified as a potential KE in NCC migration-inhibition. However, at present there is no direct proof. It is inevitable to perform knock-down experiments using the human NCCs to test a causal relationship. Moreover, more unspecific NCC toxicants should be tested in the Cx43 assay to investigate whether reduction of Cx43pq number is associated with migration-inhibition. If a causal role of Cx43 is confirmed, it would be of interest to find which protein function is involved, for example by testing Cx43 mutant proteins lacking specific protein binding domains.

Future perspectives
Migration is not the only NCC function. Hence, other NCC function assays could be developed that test for example for induction of NCC fate, disturbance of EMT or whether toxicants affect the differentiation potential of NCCs. Likewise, the existing follow-up assays could be throughput-optimized. Use of fluorescently labelled cells in the cell tracking assay would allow automated cell tracking and higher-throughput variants for the transwell assays can be envisaged.

As most of the neurodevelopmental disturbances have a mixed etiology, it would also be of interest to test the effect of low concentration of known NCC toxicants on NCCs with a susceptible genetic background, for example with mutations in genes related to neurocristopathies.
List of publications

Journal publications


Conference paper

Record of contributions

Manuscript 1: Design of a high-throughput human neural crest cell migration assay to indicate potential developmental toxicants
I conceived and designed the experiments in collaboration with Tanja Waldmann and Marcel Leist. I performed most of the experiments.
Heidrun Leisner differentiated the cells and performed the remaining part of the experiments. Christiaan Karreman developed the software to analyse migration, with some input from my side. Yong Jun Kim and Gabsang Lee provided the iPSC-derived NCC.
I analysed all the data. I designed the figures and wrote the manuscript in collaboration with Marcel Leist.

Manuscript 2: Combination of multiple neural crest migration assays to identify environmental toxicants from a proof-of-concept chemical library
I conceived and designed the experiments in collaboration with Xenia Dolde and Marcel Leist. Xenia Dolde and I performed all the migration experiments.
Alice Krebs performed confocal microscopy. Kevin Pinto-Gil and Manuel Pastor provided the information for the chemical space (Fig. 6). Mamta Behl provided the compound library.
I analysed almost all data, with the exception of the transwell migration data (analysed by Xenia Dolde).
I designed the figures and wrote the manuscript in collaboration with Marcel Leist.

Manuscript 3: A structure-activity relationship linking non-planar PCBs to functional deficits of neural crest cells: new roles for connexins
I conceived and designed the experiments in collaboration with Xenia Dolde, Petra Chovancova and Marcel Leist. I performed most of the experiments.
Petra Chovancova performed immunofluorescent stainings, confocal microscopy and the incorporation of labeled sugars. Xenia Dolde performed the transwell migration experiments. Anna-Katharina Holzer measured neurite outgrowth of peripheral neurons. Vladimir Purvanov, Ilona Kindinger and Daniel F. Legler did all experiments with the peripheral blood lymphocytes (Fig. 3B). David Higton, Steve Silvester and Richard Maclellan analysed the PCB distribution in the well (Fig. 2). Barbara MA van Vugt-Lussenburg and Bart van der Burg performed the CALUX assays and analysis. Enrico Glaab provided information for the chemical properties.
I developed the algorithms to quantify gap junctional communication and Connexin43 plaques and analysed almost all data. I designed the figures and wrote the manuscript in collaboration with Marcel Leist.
Acknowledgement

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Meinem Thesiskomitee bestehend aus Prof. Dr. Thomas Brunner und Prof. Dr. Daniel Legler möchte ich für anregende Diskussionen bedanken.

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Mein Dank geht auch an meine ehemaligen Bachelorstudenten Yi-Guang, Katharina und Oliver sowie meinen VTK-Student Markus, welche mir bei meinen Experimenten geholfen haben und durch die ich sehr viel gelernt habe.

Ich bedanke mich bei meiner Familie und all meinen Freunden, welche mich auf meinem bisherigen Weg begleitet haben.

Zu guter Letzt danke ich Christoph für seine stetige Unterstützung, Diskussionen über (Un)Sinn der Forschung, wöchentliche Wanderausflüge und das Mitmachen all meiner Hochs und Tiefs.
List of references


List of references


List of references


List of references


List of references


OECD (2016). Report on Integrated Testing Strategies for the identification and evaluation of chemical hazards associated with the developmental neurotoxicity (DNT), to facilitate discussions at the Joint EFSA/OECD Workshop on DNT. Tech. rep., OECD.


Supporting Information

Nyffeler et al.:

**Design of a High-Throughput Human Neural Crest Cell Migration Assay to Indicate Potential Developmental Toxicants**

**Supplementary Data**

Tab. S1: List of antibodies used in this study

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Tab. S2: List of primers used in this study for quantitative RT-PCR

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**Differentiation of NCC from iPSC**

The differentiation of NCCs from iPSC (IMR90) cells was performed by dual SMAD inhibition instead of the MS5-induction step, following exactly our recently published protocol (Mica et al., 2013; Chambers et al., 2016). At day 11 of differentiation, cells were re-plated on poly-L-ornithine/fibronectin/laminin coated dishes and expanded and cryopreserved exactly as the hESC-derived NCC.

Alternatively, iPSC were generated from the human fibroblast line GM02036. Sendai virus was used as non-integrating vector to introduce the Yamanaka factors (Invitrogen) as described earlier (Choi et al., 2014). In these iPSC (GM02036) cells, the silencing of ectopic genes (Yamanaka factors) was confirmed by PCR. Pluripotency was confirmed by measurement of protein expression of Nanog, Oct4, and SSEA3/4 at passage 12. Differentiation of NCCs from iPSC (GM02036) cells was performed using the rosette induction protocol with a small modification: instead of N2S medium, Neurobasal medium supplemented with N2, B27 without vitamin A (both from Gibco), L-glutamine and EGF/FGF2 was used. The same medium composition was used for the cMINC assay using these cells.

**Time-lapse video microscopy to assess cell proliferation and cell tracking**

The time-lapse experiment was performed using an Axio Observer.Z1 microscope (Zeiss), equipped with an Axiocam MRm camera and a live-cell chamber (equilibrated at 37°C and 9% CO₂). Phase contrast images of the migration zone were acquired every 10 min using a 5x objective. Afterwards, cells were stained with calcine-AM and H-33342 and migration as well as viability were quantified exactly as described in “NCC migration setup” (manuscript main part) to ensure that the toxicants worked as under standard cMINC conditions with exposure in a standard incubator.

To assess cell proliferation in the migration zone, a circle of radius 1 mm was drawn manually by an experienced person on the time-lapse images to define the ROI. Cell divisions in this ROI were manually counted, supported by a Fiji (Schindelin et al., 2012) script provided by Martin Stöckl (Bioimaging Center of the University of Konstanz (BIC)). The experimenter was blind to the respective treatments. The number of counted cell divisions was normalized to the number of migrated cells in the same well as measured with the H-33342 and calcine staining. The time-lapse experiment was performed once with three technical replicates for untreated, CytoD and FBS and with two technical replicates for taxol, LiCl, CdCl₂ and AraC.

For cell tracking experiments, cell tracking was performed manually using the ImageJ plugin “Manual Tracking”. For each biological replicate, ten individual cells were traced per condition (and 20–30 cells for the untreated control). The cumulative distance of each cell was normalized to the median of the untreated control. Statistical significance was calculated on the combined data of three biological replicates using a Wilcoxon rank sum test to compare treatments to the untreated control.

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Fig. S1: Characterization of H9-derived NCCs
(A) Graphical representation of the differentiation procedure to obtain NCCs from human pluripotent stem cells. In a first step, cells were differentiated on MS-5 feeder cells for 20 days to a rosette stage. The rosettes were picked and differentiated further for seven days (day 0’-7’). Then, the target population was obtained by FACS sorting (HNK-1+/Dll- cells), and was further expanded for up to 30 days before freezing (days 0’’-30’’). (B) The expression of the NCC marker HNK-1 was monitored by FACS analysis during the expansion phase (days 7’’-21’’); similar data were also obtained after thawing of a frozen sample. (C) Representative 2D expression plot obtained by FACS analysis of cells after thawing: about 95% of all cells expressed either of the two NCC markers HNK-1/p75; 70-80% of the cells were double-positive. (D) NCCs were thawed and seeded under standard assay conditions. After 24 h, cells were fixed and stained for GFAP (left) and Pax6 (right). The scale bar corresponds to 100 µm. Note that in the two images no positive cells are shown, as they were usually not evident under our culture conditions. Both antibodies were used in parallel on central neural precursor cells (Pax6) and astrocytes (GFAP), and they worked well under our staining conditions.
Fig. S2: Differentiation of iPSC into NCCs and use in the cMINC assay

(A) To obtain NCCs, iPSC (IMR90) cells were differentiated via dual SMAD inhibition for 11 days towards Sox10 positive NCCs as described in Mica et al. (2013) and further expanded for two weeks in N2-S medium supplemented with EGF and FGF2 before freezing. After thawing, cells were seeded on glass for 24 h before they were fixed and labelled with immunofluorescent antibodies against p75 and counterstained by H-33342. The scale bar corresponds to 50 µm. (B) The cMINC assay was performed using these iPSC (IMR90)-derived NCCs under optimized assay conditions (24 h toxicant exposure; see also Fig. 4). Concentration-response curves were obtained for three exemplary compounds. All values are normalized to untreated controls. Data are means ± SD from three different experiments. (C) Graphical representation of the procedure to obtain NCCs from human fibroblasts via iPSC. (D) Preliminary data of iPSC (GM02036)-derived NCC under optimized cMINC assay conditions (as in B). All values are normalized to untreated controls. Data are means ± SD of 4 technical replicates from one experiment.
Fig. S3: Initial testing of assay performance under conditions used earlier for MINC scratch assays (48 h exposure)
(A) A graphical representation of the assay procedure informs on the exposure scheme and test endpoints: cells (plated at 30,000/well) were treated for 48 h with compounds, followed by measurements of viability and migration at the end of the exposure period. Concentration-response curves were obtained for selected compounds, and all values were normalized to untreated controls. (B) Examples for positive control compounds; (C) Endpoint-specific controls for increased migration (FBS) and for the role of cell proliferation in the assay (mitotic inhibitor: AraC); (D) Exemplary test compounds from the class of environmental toxicants (acrylamide) and drugs (LiCl). The light gray dotted line indicates the 100% y-axis value for easier reading of the diagrams. The other two dotted lines are drawn at 90% (indicating threshold for reduced viability) and at 75% (indicating threshold for reduced migration). Data are means ± SD from three different experiments. Statistical evaluations were not performed; data are intended for visual overview.
Fig. S4: Characterization of cell proliferation under conditions used earlier for MINC scratch assays (48 h exposure)

Cells were seeded as in Fig. 2 under standard cMINC assay conditions, and incubated for 48 h with 10 µM EdU. Then, cells were stained for EdU incorporated into the DNA of proliferating cells (red), and co-stained with H-33342 (green). (A) In a representative picture double-positive cells are seen as yellow/orange. (B) The fraction of cells that had replicated their DNA within 48 h (EdU incorporation) was quantified by counting (means ± SD, n = 8). (C) Cells were treated with LiCl (10 mM), As2O3 (1 µM), CdCl2 (1 µM) or fetal bovine serum (FBS, 1%) for 48 h and EdU incorporation was assessed. Data were normalized to untreated control cells; (D) A migration experiment (as described in Suppl. Fig. S3A) was performed, and cells in the migration zone were imaged every 10 minutes using time-lapse video microscopy. The number of cells arising from mitosis in the circular region of interest (ROI) was determined from evaluation of the video tracks. For control cells, 185 ± 17 mitoses occurred. This number was normalized to the number of cells in the ROI at the end of the assay. Under control conditions, 442 ± 35 cells migrated into the ROI (e.g., 42.7% ± 5.1% of cells in the ROI were generated by proliferation instead of migration from the outside); * p < 0.05; ** p < 0.01 (conditions with inhibited proliferation are highlighted in red).
Fig. S5: Time-lapse video microscopy for cell tracking in the new cMINC assay format
NCCs were thawed and plated at 30,000/well. After one day, silicone stoppers were removed and medium was changed. Another 24 h later, treatment with CytoD (200 nM), taxol (10 nM), FBS (4%) and AraC (1 µM) was initiated. Cell behavior was recorded for 24 h by phase contrast time-lapse video microscopy. (A) Representative still images from the video sequence are shown for selected time points. The width of one micrograph corresponds to 1 mm. The dashed line indicates the approximate position of the migration zone. Note that only the lower left quarter of the total image (and migration zone) is shown. (B) For each treatment condition, ten individual cells were traced, and for each control about 20-30 cells were traced. For each cell, the total distance of movement along its migration track was measured. All data were normalized to untreated controls in the same experiment. As the box-and-whisker plot displays data of three different experiments, each box represents the results of 30 (treatment) or 80 (controls) tracked cells. The box represents the first and third quartiles, the black line the median (number given in the black square) and the whiskers are at an interquartile range of 1.5. CytoD, cytochalasin D; FBS, fetal bovine serum; AraC, cytosine arabinoside; *** p < 0.001, n.s.: not significant.
Supporting information references


SUPPORTING INFORMATION

Combination of multiple neural crest function assays
to identify environmental toxicants
from a proof-of-concept chemical library

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1. List of antibodies used in this study

<table>
<thead>
<tr>
<th>Target Name</th>
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<th>Host</th>
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2. List of toxicants used in this study

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<td>Sigma</td>
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### Supporting information Fig. S1. Overview of the screen library.

All library compounds were grouped into one of six chemical classes. The compounds marked with color were identified as positive hits in the hit confirmation testing. They comprise 10 of 12 flame retardants, 7 of 17 pesticides and 6 of 15 drug-like compounds whereas all polycyclic aromatic hydrocarbons, industrial chemicals and negative controls were identified as 'negatives'.

<table>
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<tr>
<th>Flame Retardants (12)</th>
<th>Pesticides (17)</th>
<th>Drug-like Compounds (15)</th>
<th>Industrial Chemicals (9)</th>
<th>Negative Controls (5)</th>
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<td>Organophosphate (8)</td>
<td>Organochlorines (5)</td>
<td>1-Methyl-4-phenylpyridinium iodide (MFP+)</td>
<td>2-Methoxyethanol</td>
<td>Acetyl salicylic acid</td>
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<td>1-Ethyl-3-methylimidazolium diethylphosphate</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
<td>5-Fluorouracil</td>
<td>3,3'-Imidodipropionitrile</td>
<td>Acetaminophen (4-hydroxyacetanilide)</td>
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<td>Dichlorodiphenyltrichloroethane (DDT)</td>
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<td>Acetic acid, manganese(2+) salt</td>
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<td>6-Propyl-2-thiouracil</td>
<td>Acrylamide</td>
<td>L-Ascorbic acid</td>
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<td>Colchicine</td>
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<td>Tris(cyclohexyl) phosphate (TCP)</td>
<td>organophosphates (2)</td>
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<td>Triphenyl phosphate (TPP)</td>
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<td>Diethylstilbestrol</td>
<td>Rotenone</td>
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<td>Tribis(2-chloroethyl) phosphate</td>
<td>Parathion</td>
<td>Hexachlorophene</td>
<td>Tebuconazole</td>
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**Polycyclic aromatic hydrocarbons (17)**
- 4-H-Cyclopenta[d,e,]phenanthrene
- Acenaphthene
- Acenaphthylene
- Anthracene
- Benz[a]anthracene
- Benz[a]pyrene
- Benz[b]fluoranthene
- Benz[k]fluoranthene
- Benzog[b]perylene
- Chrysene
- Dibenz[a]anthracene
- Dibenz[a]chrysene
- Dibenzo[a]anthracene
- Dibenzo[a]pyrene
- Fluorene
- Naphthalene
- Phenanthrene
- Pyrene

**PBDE (3)**
- 2,2',4,4'-Tetrabromodiphenyl ether
- 2,2',4,4',5-Pentabromodiphenyl ether
- 2,2',4,4',5,5'-Hexabromodiphenyl ether

**Other (1)**
- 3,3',5,5'-Tetrabromobisphenol A
Supporting information Fig. S2. Performance of the assay.
Cytochalasin D was run as a positive control on every assay plate. Displayed are the measured migration for cytochalasin D (in red) and the variance of the solvent control (in blue) over 35 different assay plates run in different weeks and using various cell lots.

Supporting information Fig. S3. Hit confirmation with primary assay.
Compounds identified as potential hits in the screen were re-ordered and concentration-response curves were obtained for viability (gray triangles) and migration (blue circles). All values are normalized to the solvent control (0.1% DMSO). The horizontal light gray dotted line indicates the 100% value for easier reading of the diagrams. The other two gray lines are drawn at 90% and 75% to indicate the threshold for reduced viability and migration, respectively. A log-logistic function with constraints was fitted to the concentration-response curve and the EC90 of viability and the EC75 of migration were extrapolated. The ratio between these two values was termed ‘specificity’, whereas ‘efficiency’ was defined as the amount of migration-inhibition at the EC90 of viability.

(A) Concentration-response curves from additional confirmed hits from the group of pesticides and drug-like compounds. Data are means±SD from three experiments.
(B) Non-confirmed hits, i.e. compounds where at the highest tested concentration (displayed in brackets) no migration-inhibition occurred. Data are means±SD from two experiments.
Abbreviations: BPA: bisphenol A; Ac: acenaphthylene; Dac: dibenz[a,c]anthracene.
Supporting information Fig. S4. Overview of the procedure to fit concentration-response curves for viability data.

The data of three independent experiments were averaged and fitted with log-logistic functions with different constraints and fixed values. Using a likelihood-ratio test, it was tested whether it was justifiable to use a function with three degrees of freedom (curves 2 and 3) over the one with two degrees of freedom (curve 1, fixed values at 0 and 100%).
Supporting information Fig. S5. Overview of the procedure to fit concentration-response curves for migration data.

The data of three independent experiments were averaged and fitted with log-logistic functions with different constraints and fixed values. In a first step, it was tested whether the data indicate an increase in migration (curve 4). If this was not the case, a likelihood-ratio test was performed to test whether it was justifiable to use a function with three degrees of freedom (curves 2 and 3) over the one with two degrees of freedom (curve 1, fixed values at 0 and 100%).
Supporting information Fig. S6. Retesting of confirmed hits under conditions that prevent proliferation.
(A) All confirmed hits were retested in the cMINC assay under conditions that prevent proliferation (addition of 1 µM cytosine arabinoside (AraC) during the toxicant exposure). Compounds were tested at a single concentration (the EC90 or the highest non-cytotoxic concentration if the EC90 was not reached). All results were normalized to the control (1 µM AraC, 0.1% DMSO). Data are means±SD from at least two experiments. (B) Representation of the results in absence of proliferation (+ AraC) compared to the efficacy from the hit-confirmation testing (- AraC).
Abbreviations: CytoD: cytochalasin D; TB-BPA: 3,3′,5,5′-tetrabromobisphenol A; 6-OHDA: 6-hydroxydopamine hydrochloride; DES: diethylstilbestrol.
Supporting information Fig. S7. Summary of the cMINC assay results.

(A) Schematic indicating the potencies of the confirmed hits displayed as EC90 of viability and EC75 of migration. Compounds with high specificity have a steep connection line. *: compounds for which the highest tested concentration was not cytotoxic; **: EC values for rotenone and colchicine were multiplied with 10 and 100, respectively, to fit on the scale. (B) List of all confirmed hit compounds ordered from high specificity (top) to low specificity (bottom). (C) List of all confirmed hit compounds ordered from high efficacy (top) to low efficacy (bottom).

The color code represents the chemical classes: green: flame retardants; blue: pesticides; pink: drug-like compounds.
Supporting information Table S1. Summary of the results from all assays.

Quantitative results from all four migration assays used in this study. For the standard assay (cMINC), the EC90V (EC90 of viability) and EC75M (EC75 of migration) were obtained by curve fitting of the concentration-response data. If the fitted value was above the highest tested concentration, the '>' sign was introduced. ‘Specificity’ was defined as the ratio of EC90V and EC75M, whereas ‘efficacy’ was defined as the migration-inhibition at the fitted EC90V. For all other assays, compounds were tested at the EC90V of the cMINC assay or at the indicated concentration. The efficacy refers to the migration-inhibition of this test concentration.

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<th>EC75M [µM]</th>
<th>Specificity</th>
<th>Efficacy</th>
<th>cMINC + AraC</th>
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<th>Cell Tracking</th>
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<td>29%</td>
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<td>49%</td>
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<td>57%</td>
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<td>47%</td>
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Supporting Information XX
SUPPORTING INFORMATION

A structure-activity relationship linking non-planar PCBs to functional deficits of neural crest cells: new roles for connexins

Johanna Nyffeler\textsuperscript{1,2}, Petra Chovancova\textsuperscript{1,3}, Xenia Dolde\textsuperscript{1,3}, Anna-Katharina Holzer\textsuperscript{1}, Vladimir Purvanov\textsuperscript{4}, Ilona Kindinger\textsuperscript{4}, Anna Kerins\textsuperscript{5}, David Higton\textsuperscript{5}, Steve Silvester\textsuperscript{5}, Barbara MA van Vugt-Lussenburg\textsuperscript{6}, Enrico Glaab\textsuperscript{7}, Bart van der Burg\textsuperscript{6}, Richard Maclennan\textsuperscript{5}, Daniel F. Legler\textsuperscript{1,4}, Marcel Leist\textsuperscript{1,2,3}

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1. List of PCBs used in this study

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<td>41464-47-5</td>
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<td>2437-79-8</td>
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2. List of other toxicants used in this study

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<td>RyR</td>
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<td>EDNRb</td>
<td>NM_000115.3</td>
<td>EDNRB_1F</td>
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4. List of antibodies used in this study

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<td>(University of Oslo)</td>
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5. Mass spectrometric and gas chromatography conditions

a) Mass spectrometric conditions

| Instrumentation: | Waters Xevo QTof G2-S, Agilent 7890B, CTC PAL LHX-xt autosampler |
| Corona Discharge: | 3 µA |
| Polarity:         | positive ion |
| Cone gas:         | 100 L/h |
| Aux gas:          | 140 L/h |
| Lockmass:         | Heptacosa |
| MS:               | Positive ion, m/z 50 to 800 in 0.2sec |

b) Gas chromatography conditions

| Column:          | 30m x 0.25mm, 0.25µm Agilent DB-1MS |
| Carrier gas:     | Helium |
| Injector:        | Splitless 280°C |
| Transfer line:   | 310°C (constant pressure 60psig N2) |
| Oven:            | 100°C (0.5 min), ramp at 70°C/min to 200°C, then ramp at 40°C/min to 300°C, then ramp at 25°C/min to 345°C. |
| Flow:            | 1.2mL/min |

6. Gene expression study with qPCR

Cells were seeded in 6-well plates at the same cell density as in the migration assay and cultured under standard assay conditions (i.e. medium change after one day and harvesting after 3 days in culture). PCB treatment was added 24 h before harvesting. Cells were lysed in 1 ml PeqGOLD Trifast™ (PEQLab, Erlangen, Germany). Total RNA was isolated by phenol-chloroform extraction and reverse transcribed, exactly as described in Nyffeler et al. (2017). The typical RNA yield was 3 µg per preparation. Quantitative real-time PCR was carried out using exactly 1 µl of the cDNA solution (with a concentration in the range of 0.1-1 µg/ml), 5 µl SsoFastTM EvaGreen® Supermix (Bio-Rad), 200 µM of each forward and reverse primer in a total volume of 10 µl. Primers used are listed in the Supplementary Information or in (Nyffeler et al. 2017) (primers for cadherins, integrins and GAPDH). Amplification was carried out in 96-well plates on a CFX96™ Real-Time PCR Detection System (Bio-Rad). Samples were heated for 2 min to 98°C, followed by 40 cycles of 2 s at 98°C and 5 s at 60°C. A melt-curve from 75°C to 95°C in 0.5°C steps was obtained to measure presence of primer dimers or other side products. The cycle threshold (Ct) values were determined using the Bio-Rad CFX Manager™ Software v2.0 (Bio-Rad). For assessing the connexin profile of untreated NCC, results were analysed using the ∆Ct method (Livak and Schmittgen 2001), i.e. Ct values of the genes of interest were compared to Ct values of the reference gene GAPDH. For assessing gene expression changes upon PCB treatment, the ∆∆Ct method was used, i.e. the ∆Ct values of each gene were compared among PCB-treated samples and one untreated control. Each experiment was performed using two untreated controls (and optional solvent control) and data were normalized to one untreated control. Thus, the untreated sample in the graph represents the other untreated control.
7. Incorporation of sialic acids into cell membrane glycoconjugates

This method builds on the capacity of cells to recognize the azide-modified variant of mannosamine (ManNAz) instead of mannosamine (Mahal et al. 1997). In cells, this sugar is further metabolized to sialic acid, which is then detected by the described method by coupling of the azide group to biotin, and subsequent fluorescent detection (Agard et al. 2004; Ning et al. 2008; Spate et al. 2014). Tetraacetylated sugar is used to allow for cell permeation. The acetyl groups are removed by cellular esterases. In summary, detection of sialic acids on the surface of the cells means, that the precursor sugar has been metabolized, then incorporated in glycoproteins (and lipids), processed in the Golgi and transported to the cell membrane.

Cells were seeded (95,000 cells/cm²) in 315 µl medium/well in PLO/fibronectin/laminin coated 8-well µ-slides with glass bottom (iBidi, Munich, Germany). After one day, medium was exchanged for fresh medium (320 µl). After another day, test compounds were added (40 µl of a 10x concentrate, i.e. 80 µM concentrate for PCBs). Three hours later, 100 µM tetraacetylated mannosamine with an azide group at C2 (Ac₄ManNAz) sugar precursor (provided by V. Wittmann, University Konstanz, Germany) were added (40 µl of a 10x concentrate) for 21 h. Afterwards, cells were washed and reacted for 20 min with 100 µM dibenzocyclooctyne (DBCO)-biotin (Jena Bioscience, Germany) followed by 8 µg/ml streptavidin-AlexaFluor488 conjugate (Life Technologies, US) and 1 µg/ml Hoechst-33342 for 15 mins. Images of live cells were obtained with a laser scanning confocal microscope LSM 880 (Zeiss, Germany) equipped with gallium arsenide phosphide (GaAsP) detector, using a 40x/NA 1.40 oil objective. Z-stack images of 13 slices spanning a range of 6 µm in total were acquired. Quantification was performed on the maximum intensity projection images by measuring the number of pixels brighter than 0.1, on a normalized 0→1 scale, and normalized to the solvent control.
Supporting information Fig. S1. Incorporation of labeled sialic acids into cell membrane glycoconjugates upon PCB treatment.

(A) NCCs were pretreated with toxicants for 3 h, before chemically-tagged sialic acid precursor (Ac₄ManNAz) was added. Live-cell labeling of modified sialic acids and their subsequent imaging was performed 24 h after toxicant addition. (B) Representative maximum intensity projection images of cells exposed to the indicated toxicants and labeled for incorporated sialic acids (green). Nuclei were stained with Hoechst-33342 (red). Quantification data are means±SD of 2 biological experiments.

Supporting information Fig. S2. Gene expression levels upon PCB180 treatment.

NCCs were treated with 10 µM PCB180 for 24 h or left untreated. Then, cells were harvested, RNA isolated and reverse transcribed. Expression levels of (A) differentiation markers, (B) cadherins and (C) integrin subunits were assessed by RT-qPCR. Data are normalized to the untreated control and expressed as means±SEM from 3 biological experiments. None of the changes observed were statistically significant. Abbreviations: EDNR: endothelin receptor; THR: thyroid hormone receptor.
Supporting Information Fig. S3. Gene expression changes triggered by PCBs in NCC.
NCCs were treated with 10 µM PCB for 24 h. Then, cells were harvested, RNA isolated and reverse transcribed. Data are normalized to an untreated control and are expressed as means±SEM from 3 biological experiments. Abbreviations: RA: retinoic acid; RAR: retinoic acid receptor; RXR: retinoid x receptor; VDR: vitamin D receptor.
Supporting information Fig. S4. Concentration-response curves of non-, mono-, tri-, and tetra-ortho chlorinated PCBs measured using the standard cMINC assay.

NCCs were treated with different concentrations of PCBs. After 24 h, cells were live-stained and evaluated for migration (colored circles) and viability (gray triangles). The horizontal gray dotted lines indicate the 100% (control level) and 90% (threshold for cytotoxicity) value. Data are expressed as means±SD from 3 independent biological experiments.
Supporting information Fig. S5. Concentration-response curves of di-ortho chlorinated PCBs measured using the standard cMINC assay.

NCCs were treated with different concentrations of PCBs. After 24 h, cells were live-stained and evaluated for migration (colored circles) and viability (gray triangles). The horizontal gray dotted lines indicate the 100% (control level) and 90% (threshold for cytotoxicity) value. Data are expressed as means±SD from 3 independent biological experiments.
Supporting information Fig. S6. Chemical properties of the tested PCBs.
The computed hydrophobicity (n-octanol/water partition coefficient, $K_{\text{ow}}$) was obtained using the fragment-based implementation of the xLogP algorithm (Wang et al. 1997) in the Molinspiration mib toolkit (Ertl et al. 2000), and expressed as negative decadic logarithm (logP). The same program was also used to compute the molecular weight. Polar (energy required for breaking of hydrogen bonds between the water solute and PCBs) and apolar (energy gained by removing hydrophobic drug parts from water, and therefore increasing its entropy) desolvation energies were determined using AMSOL (Cramer and Truhlar 1992) and the protocol described in Wei et al. (2002). The color code corresponds to the number of ortho-chlorines. PCBs with green open circles are inactive in the cMINC assay.
Supporting information Fig. S7. Collection of literature data of PCB effects on various cellular targets. PCBs were categorized as inactive (blue) or active (red) based on the published results and compared to the cMINC assay. ‘Number of corrects’ refers to the number of inactive non-ortho PCBs and active ortho-PCBs and gives, if divided by the number of tested PCBs the % similarity.
Supporting Information Fig. S8. Activity of 12 PCBs on various receptor systems and cellular stress pathways.

For a set of 12 PCBs, concentration-response curves of various CALUX assays were obtained and the effective concentrations color-coded. EC10 values are reported for AhR, ERα, PXR, AP-1 and ESRE; EC20 values are displayed for AR and PR. Abbreviations: AhR: aryl hydrocarbon receptor; AR: androgen receptor; ERα: estrogen receptor alpha; PR: progesterone receptor; PXR: pregnane X receptor; AP1: activator protein 1; ESRE: endoplasmic reticulum stress.

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Supporting information Fig. S9. Disturbance of connexin43 localization in NCC.

(A). Representative confocal images of untreated and PCB180-treated NCC stained for nuclei, plasma membrane sialic acids, Cx43 and Golgi. Red arrows indicate Cx43 on the cellular plasma membrane (PM). 

(B). Merged images of nuclei (blue), plasma membrane sialic acids (green) and Cx43 (red) from (A) and their corresponding schemes representing Cx43 localization in untreated and PCB180-treated cells.
Supporting information Fig. S10. Gene expression levels of connexins.

(A) Connexin gene expression levels of NCCs cultured under standard conditions were measured using RT-qPCR. Data are expressed relative to expression levels of the reference gene GAPDH. For example: Cx40 is expressed approx. 1000-fold less than GAPDH. Data are from two biological experiments with the exception of Cx43 (N=7) and Cx45 (N=1). Not exp.: not expressed in NCC, (but primer worked in other cell lines); n.d: not detected with at least 2 primer pairs. (B) Cx43 expression levels upon treatment with PCBs. NCCs were treated with 10 µM PCB153 or PCB180 for 24 h. Then, cells were harvested, RNA isolated, reverse transcribed and expression levels assessed by RT-qPCR. Data are normalized to an untreated control and are expressed as means±SEM from 3 biological experiments. N.d.: not detected with two different primer pairs.
## Supporting Information Table S1. Summary of all structure-activity results.

| PCB group | Congener | chlorine positions | α | e | m | p | t | β | 2,2',3,3'-t | 2,2',3,4,4'-t | 2,2',3,4,5,5'-t | 2,2',4,4',5,5'-t | 2,2',3,3',4,4',5,5'-t |
|-----------|----------|-------------------|---|---|---|---|---|---|------------|------------|------------|------------|------------|------------|
| PCB 126  | 3,3',4,4'  | 0 3 2 5           | 328.44 | 9.1 | 1.05 | 4.08 | - | 23.9 | 90.0 | 95.7 | 10 | + | 52.1 |
| PCB 126  | 3,3',4,4,5  | 0 3 2 6           | 360.88 | 7.51 | 10.79 | 4.68 | - | 43.0 | 90.0 | 62.9 | 10 | - | 94.2 |
| PCB 126  | 3,3',4,4,5,5  | 0 4 2 6           | 360.88 | 7.51 | 10.79 | 4.68 | - | 43.0 | 90.0 | 62.9 | 10 | - | 94.2 |

### Supporting Information

| Supporting Information of Manuscript 3 |

Structure-activity relationship results from chemical properties and all three assays used in this study. For the migration assay (cMINC), EC90V (EC90 of viability) and EC50M (EC50 of migration) were obtained by curve fitting of the concentration-response data. If the fitted value was above the highest tested concentration, the ‘+’ sign was introduced. For gap junction intercellular communication and connexin localization, the tested concentration is indicated.

### Supporting Information – References List


XXXV